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THE SINGLE CYSTEINE RESIDUE ON AN ALPHA FAMILY CHICK LIVER GLUTATHIONE S-TRANSFERASE CL 3-3 IS NOT FUNCTIONALLY IMPORTANT

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Summary: Chick liver glutathione S-transferase CL 3-3, expressed using a baculovirus system in *Spodoptera frugiperda* (SF9) cells, contains a single cysteine residue per subunit. This enzyme was modified with iodoacetamide. Amino acid analysis indicates that 0.85 ± 0.10 cysteine residue was modified per enzyme subunit. GST CL 3-3 modified with iodo[^{14}C]acetamide was further digested with trypsin and the isotope-labelled fragments were isolated. The fragment containing the cysteine residue accounts for 53% of the total labels. The S-carbaminomethylated protein retains the glutathione conjugating activity. Therefore, the cysteine residue is not essential for the enzymatic activity of CL 3-3.

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

Cytosolic GSTs from mammalian and avian sources have been well characterized and documented. At least seventeen distinct subunits have been identified in human, rat and chick tissues, and various combinations of these subunits result in more than eighteen homo- or heterodimeric GSTs [1, 3, 4]. These isoenzymes have been classified into four non-homologous multigene families, namely, α, μ, π and θ [5, 6].

For each GST subunit, there is a binding site for glutathione and a separate binding site for the electrophilic substrate. These binding sites are referred to as the G- and H- site, respectively [7]. The chemical nature of the amino acid side chains

Abbreviations: GSTs, glutathione S-transferases; Cm, S-carbaminomethylated.

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located in the active sites are not known. Chemical modification methods have been applied to identify these amino acids [8-10], and sulfhydryl specific reagents have been repeatedly utilized [11-13]. Tamai et al. [13] inactivated class π GSTs with N-ethylmaleimide and identified Cys^{47} as the modified residue. Under identical conditions, N-ethylmaleimide has no effect on the activity of class α or μ GSTs. Conversely, Carne et al. [11] reported that modification of thiol groups decreased both the enzymic and binding activities of class α GSTs.

Previous studies on chemical modification of thiol residues on class α GSTs were carried out with enzymes having multiple sulfhydryl groups, which makes the interpretation of data difficult [11, 13]. Recently, we have expressed a full-length chick liver GST CL 3 cDNA clone in *Spodoptera frugiperda* (SF9) cells using a baculovirus expression system [Chang et al., in press]. This expression system has allowed isolation of large quantities of functionally active homogeneous CL 3-3 dimer of undisputed purity, without possible contamination of closely related isoenzyme(s). The expressed protein contains a single cysteine residue at position 121 of each subunit, and is an ideal candidate for studying the involvement of sulfhydryl group in the catalytic mechanism of class α GST. The expressed protein was modified with iodoacetamide, and the catalytic properties of the modified protein were characterized and compared to the wild-type enzyme.

MATERIALS AND METHODS

Iodo[^{14}C]acetamide (6.25 mM solution, specific radioactivity 0.1 mCi/ml) for protein modification was a product of New England Nuclear (Wilmington, DE, U.S.A.). Trypsin was from Boehringer Mannheim (Mannheim, FRG). All other chemicals used were reagent grade or better.

The expressed protein was purified by affinity chromatography according to Chang et al. [4]. GST activity was assayed by published methods [14-16] at 25°C. Protein concentration was determined from absorbance at 280 nm. Protein solution with a concentration of 0.77 mg/ml has 1 A_{280} unit [Chang et al., in press].

CL 3-3 (1 nmole) was modified in the dark (16 hours, 25 °C) in a solution (100 ul) containing Tris/HCl buffer (0.06 M, pH 7.9), 100 mM KCl and 1 mM iodoacetamide (1.6 uCi). Reaction was stopped by adding 2-mercaptoethanol. Final concentration of 2-mercaptoethanol in the reaction mixture was 6 mM. For enzymic assay, CL 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.

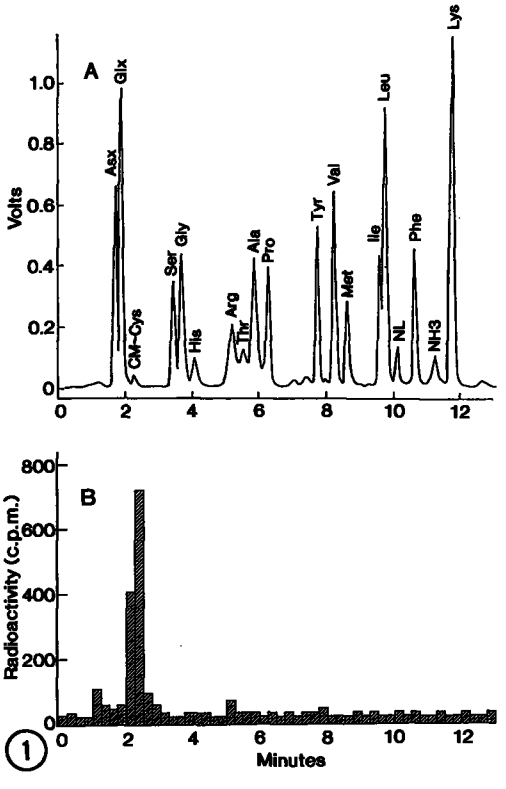
Modified proteins were digested with trypsin (24 hours, 37°C) at a substrate:enzyme ratio of 20:1 (w/w). Resulting peptides were separated on an Aquapore RP-300 C_8 reverse-phase column (2.1 X 220 mm). Elution was carried out at a flow rate of 0.3 ml/min with a linear gradient of 1% per minute of 0.08% trifluoroacetic acid in acetonitrile, and ^{14}C-labelled peptides were identified by scintillation counting.

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 analyzer [17]. Proteins for amino acid analysis were purified

on a reverse-phase column under conditions outlined above, and dried under vacuum before hydrolysing in the gas phase (24 hours, 110 °C) with 6M-HCl containing 1% (w/v) phenol. After hydrolysis, samples were analyzed in a Waters PicoTag amino acid analysis system according to the manufacturer's instructions.

RESULTS AND DISCUSSION

CL 3-3 was reacted overnight in the dark with 1 mM iodo[^{14}C]acetamide. The modified protein was purified on a reverse-phase column, then hydrolysed with 6 M HCl for quantitative amino acid analysis (Figure 1A). Based on peak area calculation, approximately 85% of the cysteine has been S-carbaminomethylated. Elutents from the column of the analyzer were also collected for scintillation counting. The radioisotope peak coincided with the modified cysteine peak, and accounted for 86% of the total labels eluted from the column (Figure 1B).



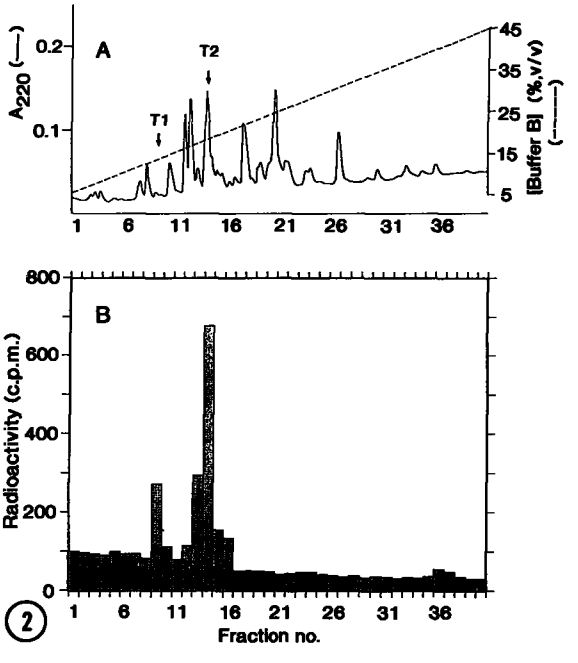


Figure 1. (A) HPLC profile of phenylthiocarbamyl amino acids generated from acid hydrolysis of ^{14}C-carbaminomethylated CL 3-3. (B) Scintillation counting of the fractions obtained in A.

Figure 2. (A) HPLC profile of tryptic digests of iodo[^{14}C]acetamide modified CL 3-3. The elution profile was monitored by UV absorption at 220 nm, and 0.3 ml fractions were collected. The peptide isolated for sequence analysis is indicated (B). Scintillation counting of the fractions obtained in A.

To ascertain that the acid hydrolysis or the chromatographic process has not destroyed or removed any modified amino acid(s), the modified protein was digested with trypsin and the labelled peptides were isolated for sequence analysis. The tryptic digests were separated on a reverse-phase column and fractions were collected for scintillation counting (Figure 2). A minor (fraction 9) and a major (fractions 13-14) radioactive peak were identified, which represented approximately 15% and 53% of the total counts, respectively.

Fraction 9 has UV absorption at 220 nm (peak T-1, fig. 2A),and was collected for sequence analysis. A sample of phenylthiohydantoin-amino acid (20 ul) was removed from each cycle of sequencing for scintillation counting. T-1 is a mixture of 2 peptides, with amino acid sequence of MESIR and YVETVRR. These two peptides represent residues 16-20 and 212-218 of the CL 3 subunit, respectively. The methionine residue is radioactive as determined by scintillation counting.

Fraction 13-14 (peak T-2, fig 2A) contains 2 peptides with sequence of QCAFVVEK and YFPAYEKVLK. They represent residues 120-127 and 132-141 of the CL 3 subunit, respectively. The Cm-cysteine phenylthiohydantoin derivative can be identified on the chromatogram of the phenylthiohydantoin analyzer (data not shown). Radioactive Cm-cysteine phenylthiohydantoin derivative was also recovered from the sequencer. Sequences of CL 3 and peptides identified after proteinase digestion are summarized in Fig. 3.

CL 3-3 was modified with iodo[^{14}C]acetamide in the presence of increasing amount of S-(dinitrophenyl)glutathione or S-hexyl glutathione. The interaction of

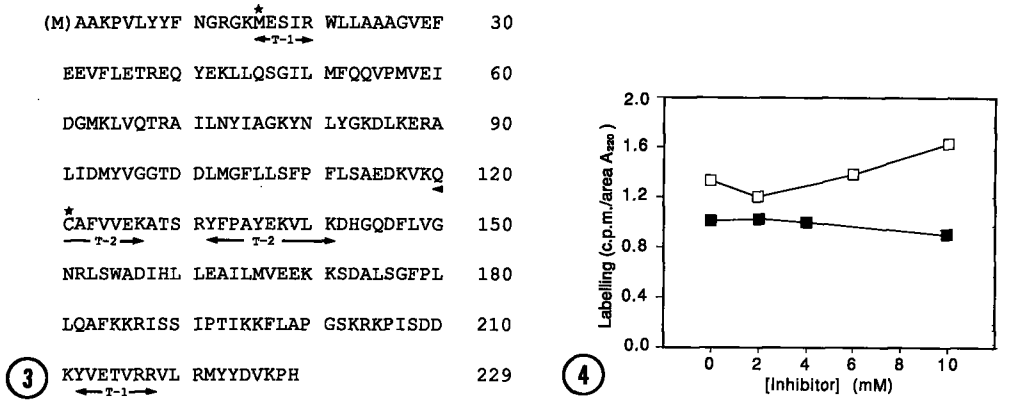


Figure 3. Amino acid sequence of CL 3 subunit and peptides identified after proteinase digestion. Radioactive amino acid phenylthiohydantoin derivatives recovered from the sequencer are indicated by asterisks (*).

Figure 4. Effect of inhibitors on iodoacetamide labelling of CL 3-3. CL 3-3 was labelled with 1 mM iodo[^{14}C]acetamide in the presence of S-hexyl glutathione (□), or S-(dinitrophenyl) glutathione (■). 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.

Table 1. Substrate Specificities of GST CL 3-3

	Specific Activity (μmol/min per mg)	
	CL 3-3	Cm-CL 3-3
1-Chloro-2,4-dinitro-benzene	6.0 ± 0.5	6.2 ± 0.4
1,2-Dichloro-4-nitro-benzene	0.023 ± 0.005	0.030 ± 0.006
1,2-Epoxy-3-(p-nitro-phenoxy)-propane	0.45 ± 0.05	0.54 ± 0.07
Δ^5-androstene-3,17-dione	N.D.	N.D.
Ethacrynic acid	2.8 ± 0.2	4.8 ± 0.1
Cumene hydroperoxide	N.D.	N.D.

N.D., not detected.
Results are the means ± S.E.M. of duplicates of at least three experiments.

these two compounds with the active site(s) of GST has been documented [19-20]. As shown in Figure 4, the presence of S-(dinitrophenyl)glutathione or S-hexyl glutathione cannot protect CL 3-3 from iodoacetamide labelling. The result excludes the possibility that Cys^{121} is located near or at the vicinity of the active sites.

The enzymic activities of the S-carbaminomethylated CL 3-3 are listed in Table 1 with the unmodified enzyme. The results show clearly that the chemically modified CL 3-3 remains enzymically active. An increase in activity with ethacrynic acid as substrate was observed (1.7 X) and remains to be explained.

The involvement of cysteine in the function of GSTs has long been a matter of debate. Van Ommen et al. [12, 21] claimed that tetrachloro-1,4-benzoquinone is a sulfhydryl specific reagent, which can irreversibly inhibit rat liver class μ and α GSTs. Schaffer et al. [22] and Ricci et al. [23] reported that modification of thiol group(s) decreased the catalytic activity of class π GSTs. Carne et al. [11] modified three out of four thiol groups on ligandin (class α GSTs) and inactivated the enzymes. Conversely, Bhargava et al. [24] labelled the cysteine residues of ligandin in the presence of urea but the catalytic activity of the enzymes were not affected after renaturation. Tamai et al [13] also reported that N-ethylmaleimide has no effect on the activity of class α or μ GSTs.

In the present study, we chemically modified CL 3-3 with iodoacetamide, which has a single cysteine on each subunit. In the process, Cys^{121} and Met^{16} were labelled. S-(dinitrophenyl) glutathione and S-hexyl glutathione, which have been shown to interact with the active site(s) on GSTs, cannot inhibit the reaction. The modified CL 3-3 is still enzymically active. Therefore, we conclude that the sulfhydryl group is not needed for the enzymic activity of this class α GST.

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