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Identification of a highly reactive sulphydryl group in human placental glutathione transferase by a site-directed fluorescent reagent

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A fluorescent maleimide derivative, *N*-(4-anilino-1-naphthyl) maleimide (ANM), a specific probe for thiol groups, reacted with human placental glutathione transferase (GST, EC 2.5.1.18), causing a complete inactivation of the enzyme in a few minutes. The modified enzyme was denatured, alkylated and digested with (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-trypsin. The tryptic digest was analysed by HPLC and a fluorescent peptide was obtained. The sequence of this peptide allowed us, by a comparison with a well known primary structure, to assign the position 47 to the most reactive cysteine of GST enzyme.

1. INTRODUCTION

Glutathione transferases (GST, EC 2.5.1.18) are a group of dimeric isoenzymes involved in the mechanism of detoxification from endogenous compounds and xenobiotics [1]. In addition they exhibit some binding properties towards different lipophilic compounds [2]. On the basis of N-terminal sequence, substrate specificity and immunological properties three classes of cytosolic GST have been identified (class α, μ, and π) common to several mammalian species [3]. Human glutathione transferase class π is an acidic protein present in all human tissues so far investigated and it has been purified and extensively characterized from human placenta by different laboratories [4–8]. Recently, a complete primary structure has been reported, as deduced from the correspondent cDNA and the gene structure [9,10]. However, very little is known about the active site. Studies already done with the class π enzyme reported the existence of two reactive sulphydryl groups/dimer, probably located at or near the active site [11,12].

In order to identify these reactive cysteinyl residues we used a specific fluorescent probe, which strongly reacted with the sulphydryl groups of the native protein, causing a complete loss of enzymatic activity in few minutes. This modified protein was, after complete denaturation and carboxymethylation of the buried cysteine residues, digested with TPCK-trypsin and the fluorescent peptide separated by HPLC. The sequence of this peptide allowed us, by comparison with a well known complete primary structure, to assign the position of the most reactive cysteine residue in the polypeptide sequence.

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Abbreviations: ANM, *N*-(4-anilino-1-naphthyl)maleimide; GST, glutathione transferase; TPCH-trypsin, (L-1-tosylamide-2-phenylethylchloromethyl ketone)-trypsin; HPLC, high performance liquid chromatography; DTT, dithiothreitol; TFA, trifluoroacetic acid; PTH-amino acids, phenylthiohydantoin-amino acids; N.D., not detected

2. MATERIALS AND METHODS

ANM was purchased from Fluka Chemie AG, Switzerland. TPCK-trypsin was obtained from Sigma Chemical Company, USA. All other compounds were readily available commercial products.

The enzyme was purified from human placenta essentially as described elsewhere [13]. The enzymatic activity was measured at room temperature according to the method of Habig and Jakoby [14].

The reduced enzyme was dialysed against 10 mM K-phosphate buffer, pH 7, containing 1 mM EDTA. GST and ANM reacted in the same buffer at 37°C at three different molar ratios for 30 min. At fixed times an aliquot of incubation mixture was taken for measuring the residual activity. The reaction was stopped by adding 100 μl of 100 mM DTT.

Reduction and carboxymethylation of the modified enzyme was carried out according to a procedure described elsewhere [15].

S-carboxymethylated GST was digested with TPCK-trypsin in 0.1 M ammonium bicarbonate, pH 8 at 37°C for 24 h.

HPLC purification of peptides was carried out with a Beckman System Gold (CA, USA), equipped with a Beckman programmable detector Module 166 and a Shimadzu (Kyoto, Japan) RF-530 fluorescence monitor, connected in tandem.

Automated Edman degradation of the fluorescent peptide was carried out using an Applied Biosystems model 470 A gas phase sequencer equipped with an Applied Biosystems model 120 A PTH-analyzer.

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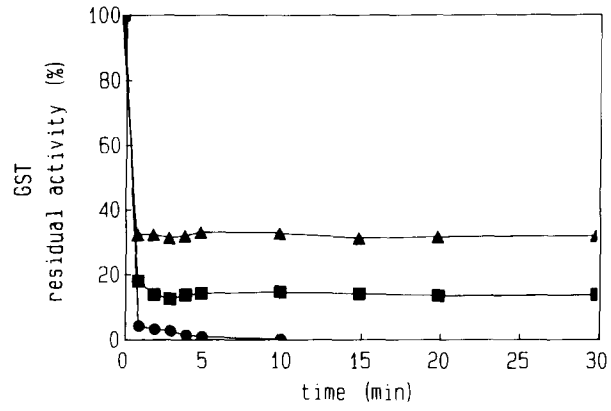

Fig. 1. Time course of GST inactivation by ANM. GST and ANM reacted, as described in section 2, at the following molar ratios: 1:5 (▲), 1:10 (■), 1:50 (●). A complete inactivation of the enzyme was observed in 10 min when GST was incubated with 50 molar excesses of ANM.

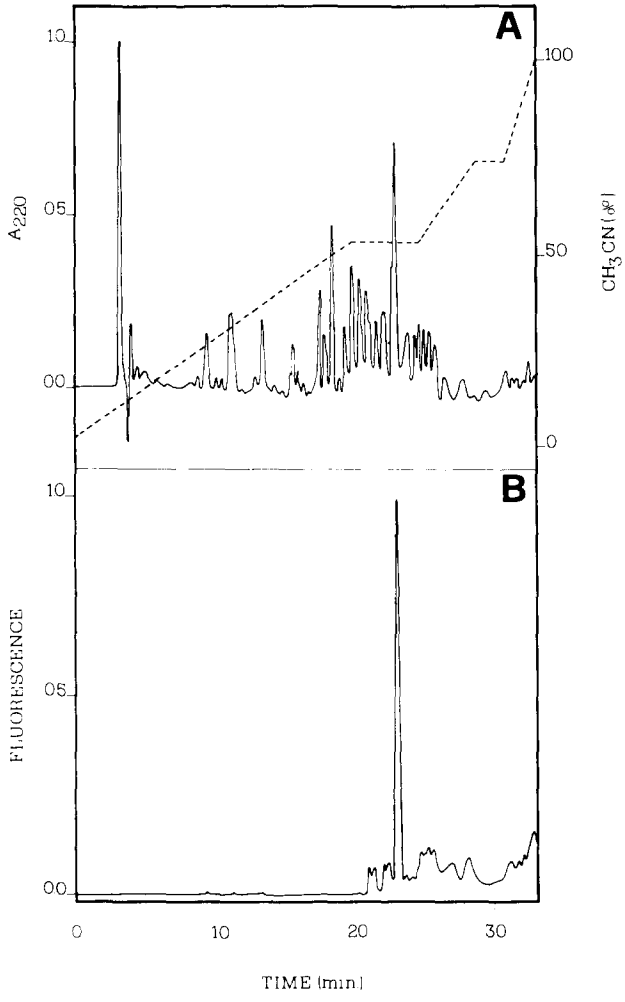

Fig. 2. HPLC pattern of TPCK-trypsin digest of ANM labelled enzyme. Elution was carried out using a column (250 × 4.6 mm I.D.) reverse phase Aquapore RP-300 (Applied Biosystems, USA). The gradient system was composed of 0.2% TFA (solvent A) and 70% acetonitrile in 0.2% TFA (solvent B). The flow rate was 1 ml/min. Peptides were detected by monitoring their absorbance at 220 nm (A) and their fluorescence at 448 nm (B).

3. RESULTS

ANM is an N-maleimide derivative which modifies selectively the thiol groups of proteins [16]. This hydrophobic probe becomes fluorescent when it reacts with the SH groups, forming a stable adduct. In our conditions the ANM reacted with GST π at different molar ratio at 37°C for 30 min. A complete inactivation of the enzyme was obtained in the first 10 min when the molar ratio was 50:1 (Fig. 1). The modified enzyme was reduced, alkylated and digested with TPCK-trypsin at 37°C for 24 h. The tryptic digest was fractionated by HPLC on the reverse phase Aquapore 300 column and peptides were monitored by their absorbance at 220 nm and by their fluorescence (λ_{ex} = 355 nm, λ_{em} = 448 nm). A main peak of fluorescence was obtained (Fig. 2), suggesting that only one cysteine residue was extensively modified by ANM. This fluorescent peak was further sequenced by a gas phase sequencer. The result showed the following sequence:

Ala-Ser-N.D.-Leu-Tyr-Gly-Gln-Leu-Pro-Lys.

By comparison of this sequence with the complete primary structure it was possible to assign position 47 to this reactive cysteine.

4. DISCUSSION

Glutathione transferase from human placenta possesses four cysteine residues/subunit [9,10]. Preliminary experiments done either on horse erythrocyte GST (an acidic protein very similar to the placental GST) [11] or on the human placental enzyme [12] reported the existence of one highly reactive sulphydryl group/monomer. In order to identify which cysteine is involved in the maintenance of the catalytic activity we used a selective probe, which blocked in an irreversible manner the thiol groups and completely inactivated the enzymatic activity of the placental enzyme (Fig. 1) in few minutes. Complete digestion of the GST π enzyme was achieved only when the protein was denatured in the presence of 6 M guanidinium chloride and then alkylated with iodoacetic acid. This suggested the presence of a hydrophobic core very resistant to the proteolytic attack, even in the presence of up to 4 M urea. The analysis by HPLC of the tryptic digest showed only one fluorescent peak (Fig. 2), indicating that only one cysteine/subunit was extensively modified by ANM. Probably the other three cysteine residues/subunit are located inside the hydrophobic core and therefore are not accessible to our probe. The sequence of the fluorescent peptide confirmed the existence of only one highly reactive cysteine/subunit, which was identified as Cys-47.

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