Volume 293, number 1,2, 156–159 FEBS 10410 November 1991

Cysteine residues are not essential for the catalytic activity of human class Mu glutathione transferase M1a-1a*

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Received 17 September 1991

To investigate the possible involvement of a Cys thiol in the catalysis of the human glutathione transferase M1a-1a, we constructed mutants of this enzyme wherein the four Cys residues present in the native enzyme were replaced by Ala residues. Three mutants, one where all four Cys residues had been replaced and two mutants where three out of four Cys residues were changed into Ala, were characterized regarding their catalytic activities with three different substrates as well as by their binding of three different inhibitors. All three Cys-deficient mutant forms of glutathione transferase M1a-1a were catalytically active with the tested substrates and their binding of inhibitors, measured by I_{50}, were not significantly different from the values previously obtained for the wild-type enzyme. We therefore conclude that none of the Cys residues in this class Mu glutathione transferase are directly involved in the catalysis performed by this enzyme.

Glutathione transferase; Site-directed mutagenesis; Cysteine residue

1. INTRODUCTION

A possible role of a Cys residue in the catalytic mechanism of the glutathione transferases has been the subject of a number of investigations [1]. The fact that inactivation of some isoenzymes occurs upon treatment with thiol-alkylating agents, and the high conservation of Cys residues in the primary structures of class Pi as well as class Mu glutathione transferases, contrast with the finding that other naturally occurring isoenzymes lack Cys and recombinant proteins in which Cys residues have been replaced by other residues still retain enzymatic activity. On balance, however, the available evidence suggests that protein thiol groups do not contribute directly to catalysis, either in the class Pi or the class Mu glutathione transferases.

The class Pi glutathione transferases from four different species (man, mouse, pig and rat) contain highly conserved Cys residues (Cys^{15}, Cys^{48} and Cys^{171}). Schaffer et al. have shown that the bovine placental class Pi enzyme is inactivated by addition of iodoacetamide [2] and similar results have been presented by Ricci et al. [3] who inactivated the class Pi enzyme from equine erythrocytes by addition of different thiol-reagents. DelBoccio et al. [4], also studying the horse enzyme, showed that 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole lowered the activity by reacting with a residue corresponding to Cys^{48} in the human isoenzyme. Hirrell et al. [5] have shown that the human class Pi enzyme in red blood cells is sensitive to alkylation by *N*-ethylmaleimide and Tamai et al. [6], using the same alkylating reagent, got similar results when comparing the isoenzymes from rat, mouse and man. Peptide sequencing of the rat protein revealed Cys^{48} to be the alkylated residue. The inactivation of these enzymes by alkylating agents seems to be of an indirect nature, since the three-dimensional structure of the porcine class Pi glutathione transferase does not contain a Cys residue in the proximity of the putative active site [7] and since the enzymes are very similar in structure.

Regarding the class Alpha isoenzymes, addition of *p*-mercuribenzoate or *N*-(dimethylamino-3,5-dinitrophenyl)maleimide partially inactivated rat transferase 1–2 (ligandin) [8]. Extensive *S*-alkylation of the same enzyme with iodoacetamide resulted in only 30% loss of enzymatic activity [9]. Further, at least one representative of the Alpha class is totally lacking Cys residues [10], and within this class the Cys residues have a low degree of conservation.

On the other hand, the class Mu glutathione transferases contain three Cys residues (C87, C115 and C174) that are conserved in 11 known mammalian class Mu sequences, and residue C174 is also conserved in the aligned protein sequence of the class Mu-like glutathione transferase from *Schistosoma japonicum* [11]. This very high degree of conservation indicates an essential function for these Cys residues in the class Mu glutathione transferases. The enzyme studied here, human glutathione transferase M1a-1a, has previously been shown to be inactivated by both mercaptide-forming and alkylating thiol reagents [12].

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*Glutathione transferase M1a-1a has also been named glutathione transferase μ and glutathione transferase B_1B_1.

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2. EXPERIMENTAL

2.1. *Chemicals and reagents*

DNA restriction enzymes and other DNA modifying enzymes were obtained from Boehringer-Mannheim (Mannheim, Germany), Promega Corp. (Madison, WI, USA) and Amersham International (Amersham, Bucks, UK) and were used according to the recommendations of the suppliers. Radioactive nucleotides and in vitro mutagenesis kit were supplied by Amersham International. M13mp18 DNA was purchased from Boehringer-Mannheim. *S*-Hexylglutathione was synthesized by method A as described by Vince et al. [13]. Glutathione and 1-chloro-2,4-dinitrobenzene were from Sigma Chemical Co. (St. Louis, MO, USA). Oligodeoxynucleotides were synthesized by Operon Technologies (Alameda, CA, USA). *Escherichia coli* strain XL1-Blue was obtained from Stratagene (La Jolla, CA, USA) and was cultured in growth medium prepared using tryptone and yeast extract from Difco Laboratories (Detroit, MI, USA). All other chemicals used were of highest purity available.

2.2. *Mutagenesis of glutathione transferase M1a-1a*

The cDNA encoding the glutathione transferase subunit M1a (also denoted H_b [14]) in the plasmid pKHM1a [15], together with the *Trc* promoter, was subcloned into M13mp18 and mutated in the four Cys codons at positions 78, 87, 115 and 174 in the deduced amino acid sequence (numbering including the initiator Met codon), using the mutagenesis kit supplied by Amersham International utilizing the method developed by Eckstein and coworkers [16]. The mutameric oligonucleotides were designed for Cys→Ala conversions and their structures are given in Table I.

All four mutamers ('M1C78A', 'M1C87A', 'M1C115A' and 'M1aC174A') were added to the annealing mixture to create a library of mutants that subsequently was screened by slot-blot hybridization of isolated M13mp18-derivative phage stocks, essentially as described in the mutagenesis kit manual, using the different ^{32}P-labelled mutamers as probes. The DNA insert of isolated mutants was sequenced by the method of Sanger et al. [17] in order to confirm the desired mutations and to rule out unwanted changes in the entire coding region of the cDNA as well as in the *Trc* promoter region.

The replicative form of the mutant M13mp18 derivatives was digested with *Eco*RI and *Pst*I and the fragments containing the *Trc* promoter together with the cDNA encoding subunit M1a were ligated to pKT-D digested with the same enzymes. The plasmid pKT-D is a derivative of pKK233-2 (Pharmacia-LKB Biotechnology, Uppsala, Sweden) deleted between the *Acc*I restriction sites at positions 651 bp and 2246 bp (pBR322-numbering). The recombinant plasmids were used to transform competent *E. coli* XL1-blue for expression of the mutant proteins.

2.3. *Expression and purification of Cys→Ala mutants of GSTM1a-1a*

Protein was expressed and purified by affinity chromatography [18] as previously described [15]. Purity of isolated mutant glutathione transferase M1a-1a was confirmed by electrophoresis in SDS/PAGE [19] developed by staining with Coomassie Brilliant Blue R-250. Protein concentration was determined by the method of Peterson [20].

2.4. *Physicochemical characteristics*

The apparent subunit M_r of the Cys→Ala mutants of glutathione transferase M1a-1a was determined by SDS-PAGE. The isoelectric point was determined by isoelectric focusing in precast gels (Pharmacia-LKB Biotechnology, Uppsala, Sweden) following the recommendations of the manufacturer. The chromatographic properties in a C-4 reverse phase HPLC column (Dynamax-300A, Rainin Instr. Inc., Woburn, MA, USA) were investigated using the method previously described by Ostlund Farrants et al. [21] using the following gradient: 45–60% (v/v) acetonitrile in water for 20 min, 0.1% (v/v) trifluoroacetic acid. The eluted proteins were detected by their absorption at 214 nm. Purified wild-type recombinant glutathione transferase M1a-1a was in all cases used as standard protein.

2.5. *Kinetic measurements*

The glutathione transferase activity was assayed in 0.1 M sodium phosphate buffer, pH 6.5, at 30°C with three different substrates: 1-chloro-2,4-dinitrobenzene, *trans*-4-phenylbut-3-en-2-one and 1,2-

Table I

Oligodeoxynucleotides used for creating Cys→Ala mutations in glutathione transferase M1a-1a

Amino acid no.:	74 78 82
Amino acid sequence:	N A I L C Y I A R
DNA sequence, M1a/b:	5'-...AAC GCC ATC TTG TGC TAC ATT GCC CGC...-3'
	**
Mutamer: M1C78A	3'-G CGG TAG AAC CGG ATG TAA CGG-5'
Amino acid no.:	83 87 91
Amino acid sequence:	K H N L C G E T E
DNA sequence, M1a/b:	5'-...AAG CAC AAC CTG TGT GGG GAG ACA GAA...-3'
	**
Mutamer: M1C78A:	3'-C GTG TTG GAC CGA CCC CTC TGT-5'
Amino acid no.:	111 115 119
Amino acid sequence:	L G M I C Y N P E
DNA sequence, M1a/b:	5'...CTG GGC ATG ATC TGC TAC AAT CCA GAA...3'
	**
Mutamer: M1C115A:	3'-C CCG TAC TAG CGG ATG ATT CGT-5'
Amino acid no.:	170 174 178
Amino acid sequence:	F E P K C L D A F
DNA sequence, M1a:	5'...TTT GAG CCC AAG TGC TTG GAC GCC TTC...3'
	**
Mutamer: M1aC174A	3'-A CTC GGG TTC CGG AAC CTG CGG-5'

Asterisk indicates mismatch between the mutameric oligonucleotides and the DNA sequence to be mutated. Amino acid numbering includes the initiator Met codon.

Table II

Specific enzymatic activities of Cys-depleted glutathione transferase M1a-1a mutants

Enzyme	Specific activity (μmol/min · mg)		
	Substrate		
	CDNB	tPBO	NPEP
M1a-1a	174*	0.3*	0.2*
M1a-1a (C87A, C115A, C174A)	130	0.3	–
M1a-1a (C78A, C87A, C115A)	129	0.3	–
M1a-1a (C78A, C87A, C115A, C174A)	98	0.1	0.2

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; *t*PBO, *trans*-4-phenylbut-3-en-2-one; NPEP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane. The values are means of six independent measurements.

*Values from [15].

epoxy-3-(*p*-nitrophenoxy)propane using the concentrations of glutathione and electrophilic substrate previously described [22].

The sensitivity towards the inhibitors *S*-hexylglutathione, Cibacron Blue F3GA and bromosulfophthalein was tested by the procedure of Tahir and Mannervik [23].

3. RESULTS AND DISCUSSION

To directly investigate if the Cys residues in the human glutathione transferase M1a-1a (also named μ and H_bH_b) are involved in the catalytic action of this enzyme, mutants were created in which the Cys residues present in the wild-type sequence were replaced by Ala residues (Table I). The present study addresses the basic characterization of three different Cys→Ala mutants of glutathione transferase M1a-1a (Table I); one mutant M1a-1a(C78A,C87A,C115A,C174A), lacking all four Cys residues that are present in the wild-type protein, and two mutants in which three of the four Cys residues are replaced by Ala residues, M1a-1a (C87A,C115A,C174A) and M1a-1a(C78A,C87A, C115A).

The Cys-deficient recombinant enzymes were purified to homogeneity and their physicochemical properties studied. In every respect the mutant proteins were indistinguishable from their parental counterpart; the apparent M_r (26 700), isoelectric point (6.2) and elution from a reverse phase C-4 HPLC column were identical to the wild-type glutathione transferase M1a-1a (results not shown).

The specific enzymatic activities determined with three different substrates show that none of the four Cys residues present in the glutathione transferase M1a-1a are essential for catalysis; the Cys-depleted mutants were active with all substrates tested, even though the activity was somewhat reduced with 1-chloro-2,4-dinitrobenzene as compared to the wild-type enzyme (Table II).

The I_{50} values obtained with three different inhibitors were very similar to the values previously obtained for the wild-type recombinant glutathione transferase M1a-1a [15] indicating unperturbed binding sites for the inhibitors tested (Table III). One of the inhibitors, *S*-hexylglutathione, is a substrate and product analog and is considered to bind to the active site of the enzyme; the loss of the thiol groups of the four Cys side-chains do not appear to affect the binding of this inhibitor.

Our results are in agreement with those reported by Hsieh et al., who via site-directed mutagenesis have shown that the iodoacetamide-reactive thiol of Cys^{87} is not essential for the catalytic activity of the rat class Mu glutathione transferase 3–3 [24]. In contrast, studies on the same enzyme by Adang et al. [25] imply a Cys thiol at or close to the active site, based on the inhibition of enzymatic activity by a phenylthiosulphonate derivative of glutathione. However, the inhibiting effect could be due to steric hindrance of substrate binding at the active site or a conformational change in the protein structure. A similar mechanism probably explains the inactivation of the class Pi enzymes by alkylation of Cys^{48}.

We conclude from the presented work that Cys residues are not essential for the catalytic action of human glutathione transferase M1a-1a or for the binding of three different inhibitors. By analogy, such residues are probably not essential for the catalytic activity of other class Mu enzymes. However, these highly conserved

Table III

Inhibition parameters for Cys → Ala mutants of glutathione transferase M1a-1a

Enzyme	Inhibitor		
	S-Hexylglutathione	Bromosulfophthalein	Cibacron blue
M1a-1a	3*	5*	0.4*
M1a-1a (C87A, C115A, C174A)	3	2	0.5
M1a-1a (C78A, C87A, C115A, C174A)	3	3	0.3

I_{50} is the inhibitor concentration resulting in a 50% loss in activity, measured under standard conditions with 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene in 0.1 M sodium-phosphate buffer, pH 6.5, at 30 °C.

*Values from [15].

residues may still have other significant biological functions yet to be uncovered.

Acknowledgements: This work was supported by grants from the Swedish Natural Science Research Council, the Swedish Council for Engineering Sciences and the Carl Trygger Foundation.

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