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Effects of Cys10 mutation to Ala in glutathione transferase from *Escherichia coli*

Hideshi Inoue [a,*], Motohiko Nishida [b], Kenji Takahashi [a]

[a] *School of Life Science, Tokyo University of Pharmacy and Life Science, Hachioji-shi, Tokyo 192-0392, Japan*
[b] *Graduate School of Pharmaceutical Science, Hokkaido University, Sapporo 060-0812, Japan*

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Abstract

The residue Cys10 in *E. coli* glutathione transferase (GST) is apparently equivalent in primary structure to the catalytic Ser residue of the class theta GST and is located near the thiol group of GSH bound to the enzyme. The mutation of Cys10 to Ala, however, increased the specific activity toward 1-chloro-2,4-dinitrobenzene at pH 6.5. This mutation increased both the k_{cat} and K_m values for GSH and affected the pH-activity profile the enzyme. The side chain of Cys10 is thought to be important for construction of the GSH-binding site and partly for lowering the pK_a of the GSH thiol, but not to be essential for the catalytic activity.

Keywords: Glutathione; Glutathione s-transferase; Catalytic mechanism; Site-directed mutagenesis

1. Introduction

Glutathione transferase (GST) (EC 2.5.1.18) is a multifunctional protein which participates mainly in metabolism of xenobiotics by catalyzing conjugation of glutathione (GSH) with a wide variety of compounds with electrophilic groups, and also in glutathione-dependent reduction of organic peroxides [1–4]. The cytosolic GSTs are known to exist in all eukaryotes and in a subgroup of eubacteria, and are grouped into evolutionally related classes called alpha, mu, pi, sigma, theta and so on.

In the catalytic mechanism of GSH-conjugation by GST, the elevation of the nucleophilicity of the thiol group of GSH by lowering its pK_a is thought to be essential. The three-dimensional structure of GST was first reported for porcine GST P1-1 complexed with the inhibitor glutathione sulfonate, whereby the hydroxyl group of Tyr7 was revealed to be located close to the S atom of the inhibitor [5]. Subsequently, the replacement of Tyr7 of human GST P1-1 with Phe by site-directed mutagenesis was shown to result in an about 100-fold decrease in catalytic activity, and that the pK_a of the thiol group of the enzyme-bound GSH was raised by about two pK_a units [6–8]. For example, the pK_a value of the thiol group of GSH bound to human GST P1-1 [7] was estimated to be 6.3, and the mutation of the catalytic Tyr residue with Phe increases the pK_a values to about 8.7. These results suggested that the hydroxyl group of Tyr7 participate in the catalysis. Very similar results were reported for the class alpha and mu GSTs [9,10]. Thus the conserved Tyr residue near the N-terminus was assumed to play an essential role in lowering the pK_a of GSH by stabilizing the thiolate form [11]. On the basis of a series of studies using X-ray crystallography and site-directed mutagenesis, this mechanism is considered to be common in the GSTs of class alpha, mu, pi and sigma [4,12,13].

The catalytic Tyr residue appeared to be conserved also in GST from *E. coli*. However, the replacement of the corresponding residue Tyr5 with Phe did not affect the enzymatic activity at all [14]. This result suggested that *E. coli* GST is different from the GSTs of class alpha, mu, pi and sigma in the structure of the catalytic site. In class theta GST from the insect *Lucilia cuprina*, Ser9 was proposed to be the catalytic residue, because the side-chain hydroxyl group of this residue is located close to the S atom of the enzyme-bound GSH [15], and because the replacement of Ser9 with Ala considerably

* Corresponding author. Fax +81-426-767153.
E-mail address: hinoue@ls.toyaku.ac.jp (H. Inoue).

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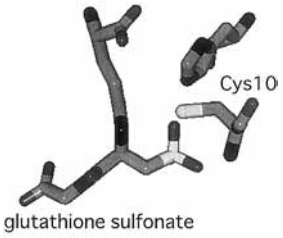

Fig. 1. Glutathione sulfonate bound to *E. coli* GST (Protein Data Bank, 1A0F) [19].

decreases the activity [16]. This Ser residue appeared to be conserved in the theta class and theta-like GSTs from eukaryotes and prokaryotes. Therefore, the conserved Ser was expected to be the consensus catalytic residue among these GSTs. In fact, the replacement of the counterpart residue Ser12 to Ala in dichloromethane dehalogenase–GST from *Methylophylus* sp. strain DM11 was shown to markedly reduce the enzymatic activity [17].

Cys10 or Ser11 in GST from *E. coli* appeared to be equivalent to the catalytic Ser residue of class theta GSTs. The crystal structure of *E. coli* GST complexed with the inhibitor glutathione sulfonate [18,19] demonstrated that the overall folding was similar to those of the eukaryotic GSTs. However, distinct differences were found in the structure of the active site. The side chain of neither Tyr5 nor Ser11 was located near the S atom of the bound inhibitor. Instead, the side-chain SH and the main-chain NH group of Cys10, and the side-chain imidazole group of His106 were found to be located close to the S atom (Fig. 1). Rossjohn et al. reported the three-dimensional structure of *P. mirabilis* GST B1-1 complexed with GSH, in which Cys10 forms a mixed disulfide bond with GSH [20]. Thus, the side chain of Cys10 in *E. coli* GST also appeared to be important for catalytic activity. In this study, the roles of Cys10 were studied by site-directed mutagenesis.

2. Results and discussion

2.1. GST activity toward 1-chloro-2,4-dinitrobenzene (CDNB)

Cys10 was replaced with Ala by site-directed mutagenesis. The specific activity toward CDNB of the resulting mutant Cys10Ala was equivalent with that of the wild-type enzyme at pH 6.5 (Table 1). However, the kinetic parameters of the mutant were significantly different from those of the wild type. The k_{cat} and K_m^{GSH} values were larger than those of the wild type nearly three and ten times, respectively, while the K_m^{CDNB} value was almost equivalent with that of the wild type. These results suggest that the side chain of Cys10 is important for GSH binding. Since the k_{cat} and k_{cat}/K_m^{CDNB} values of the mutant are larger than those of the wild type, the side chain of Cys10 is considered not to be important for catalytic mechanism.

Table 2
Specific activities toward cumene hydroperoxide

	Specific activity (μmol/min/mg)
Wild type (by Nishida [14])	0.12
Wild type (this work)	0.14
Cys10Ala	0.16

2.2. GSH-dependent peroxidase activity

GST from *E. coli* has GSH-dependent peroxidase activity toward cumene hydroperoxide [14]. The role of Cys10 in the peroxidase activity was investigated using the Cys10Ala mutant. As shown in Table 2, the mutant was as active as the wild type. Therefore, the side chain of Cys10 is not necessary for the peroxidase activity of GST from *E. coli*.

2.3. pH dependence of the activity toward CDNB

The initial velocity of GSH–CDNB conjugation by *E. coli* GST was measured at various pH values. Similar pH-profiles of the initial velocity were obtained in the presence of 5 mM GSH and 0.2 mM CDNB (Fig. 2) and in the presence of 1 mM GSH and 0.2 mM CDNB (data not shown). The initial velocity increased with increase in pH from 4.5 to 5.5, and was constant in the range of pH 5.5–9 (the data at pH above 8 are not shown). In the presence of 0.1 mM GSH and 2 mM CDNB, the initial velocity increased with increase in pH from 4.5 to 6 or 6.5 (data not shown). It is

Table 1
Specific activities toward CDNB and the kinetic parameters

	Specific activity (μmol min^{-1} per mg)	k_{cat} [a] (sec^{-1})	K_m^{GSH} [a] (mM)	K_m^{CDNB} [a] (mM)
Wild type	10.1	13	0.13 [b]	3.0 [b]
Cys10Ala	11.7	34	1.3	2.6

[a] Assay conditions: 0.05–3.0 mM GSH, 0.4–2.0 mM CDNB, 0.1 M potassium phosphate buffer (pH 6.5), and 1 mM EDTA at 30°C.
[b] The K_m values for the wild type enzyme has been corrected from those reported previously [14].

noteworthy that *E. coli* GST retains a significant activity even at pH 4.5 while other GSTs are considerably less active at this pH. At pH lower than 4.5, it was difficult to accurately measure the initial velocity due to decreased stability of the enzyme, and the activity was not detected at pH 3.5. *E. coli* GST is also active at pH as high as at least 9 (data not shown). Thus, *E. coli* GST is unique as compared with other GST values in the fact that it is considerably active in a broader range of pH. These results suggest that deprotonation of the GSH thiol is facilitated even at relatively low pH. By fitting the pH-profile of initial velocity in the presence of 5 mM GSH and 0.2 mM CDNB to the equation $y = C/(1 + 10^{pK_a - pH})$ (y, specific activity; C, constant), the pK_a value of the GSH thiol bound to the enzyme was estimated to be about 5.0. This value is lower than those obtained with other GSTs, such as human GST P1-1 [8], by more than one pK_a unit. On the other hand, the pH-profile of the activity shifted apparently to alkali by the Cys10 mutation to Ala. The apparent pK_a of the thiol group of GSH bound to Cys10Ala is about 6, which is similar to that in other GSTs. These results suggest that the side chains of Cys10 is responsible for the shift of the pH-activity profile toward acidic direction, but is not essential for catalytic mechanism at neutral pH.

3. Experimental

3.1. Materials

Synthetic oligonucleotides were obtained from Iwaki Glass Co. Glutathione and glutathione–agarose were purchased from Kojin Co. and Sigma, respectively. The other chemicals were purchased from Wako Pure Chemicals Ind.

3.2. Preparation of mutant enzymes

Site-directed mutagenesis for Cys10Ala was performed according to the procedure of Kunkel [21] using oligonucleotides TACAAACCGGGTGCCGCCTCTCTCGCTTCCC. The DNA sequences of all the mutants were confirmed using a DNA sequencer (A.L.F. DNA Sequencer II, Pharmacia). The mutations were also confirmed with the mutant proteins using a protein sequencer (model 376, Perkin–Elmer-ABI). Expression, purification and kinetic studies of the mutant enzymes were performed as described in the previous paper [14]. In brief, the mutant enzymes were expressed in *E. coli* and were purified through a GSH–agarose column to give a single protein band on SDS-PAGE.

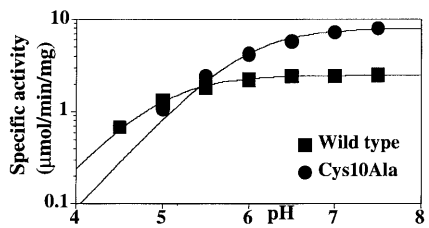

Fig. 2. Dependence on pH of the specific activities of *E. coli* GST and its mutants in the presence of 5 mM GSH and 0.2 mM CDNB at 30°C. Buffers used were as follows: 0.1 M sodium acetate for pH 4.5–5.5; 0.1 M sodium phosphate for pH 6.0–7.5. The buffers contained 3 mM EDTA.

3.3. Assay

The specific activity toward 1-chloro-2,4-dinitrobenzene (CDNB) was measured as described [22,23] at pH 6.5 in the presence of 1 mM GSH and 1 mM CDNB at 30°C unless otherwise specified. GSH-dependent peroxidase activity was determined as described [24].

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