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Active-Site Mutants of Human Glutathione *S*-Transferase P1-1: Effects of the Mutations on Substrate Specificity and Inhibition Characteristics

Hee-Joong Park, Suck-Young Yoon and Kwang-Hoon Kong*
Department of Chemistry, College of Natural Sciences, Chung-Ang University,
Dongjak-ku, Seoul 156-756, Korea

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In order to gain further insight on the relationship between structure and function of glutathione *S*-transferase (GST), the six active-site mutants, R13T, K44T, Q51A, Q64A, S65A, and D98A, of human GST P1-1 were expressed in *Escherichia coli* and purified to electrophoretic homogeneity by affinity chromatography on immobilized GSH. The active-site mutants showed marked differences in substrate specificity. The substitution of Gln51 with threonine resulted in a drastic decrease in the specific activities to <10% of the wild-type value. The substitution of Arg13 with threonine resulted in more decreased specific activity toward cumene hydroperoxide and in the I_{50} values of *S*-(2,4-dinitrophenyl) glutathione and benanstatin A. These results suggest that the substitution of Arg13 with threonine changes the conformation of the active site to increase the affinity for the product or electrophilic substrate. Lys44 seems to be in the vicinity of the H-site of hGST P1-1 or may contribute to some extents to the electrophile binding.

Keywords: Active-site residues, Glutathione *S*-transferase, Inhibition characteristics, Substrate specificity.

Introduction

Glutathione *S*-transferase (GST, EC 2.5.1.18) is a family of multifunctional proteins, catalyzing the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic compounds including alkyl- and aryl halides, epoxides, esters, and alkens (Mannervik and Danielson, 1988). Certain GSTs can also detoxify lipid and DNA hydroperoxide by their intrinsic peroxidase activity. Others catalyze the isomerization of certain steroids and play an important role in the intracellular transport of numerous hydrophobic nonsubstrate ligands such as bile acids, billirubin, and a number of drugs. The peroxidase activity is dependent on GSH, but steroid isomerase and ligandin activities are not.

GSTs are distributed in a wide range of organisms from mammals to *E. coli* (Fahey and Sundquist, 1991). GSTs can be grouped into at least four distinct classes, alpha, mu, pi, and theta, according to their structures and catalytic properties (Mannervik *et al.*, 1992). Although each isoenzyme generally exhibits a relatively broad substrate selectivity, most have unique catalytic attributes that are important in defining the role of a particular isoenzyme in the metabolism of endogenous and xenobiotic electrophiles (Armstrong, 1991). Considerable effort has been expanded to document the substrate preferences of numerous isozymes from the various gene classes and species. Among various GSTs, class Pi GSTs have attracted attention as reliable preneoplastic or neoplastic marker enzymes, the detection of which facilitates analysis of carcinogenic modifiers (Tsuchida and Sato, 1992). Moreover, their mechanisms of expression are current topics in relation to oncogene activation. Numerous studies using site-directed mutagenesis (Tamai *et al.*, 1991; Kong *et al.*, 1991; 1992a; 1992b; 1992c; 1993; 1994; Kolm *et al.*, 1992; Manoharan *et al.*, 1992a; 1992b; Nishihira *et al.*, 1992a; 1992b; Widersten *et al.*, 1992; Karshikoff *et al.*, 1993; Meyer *et al.*, 1993) and X-ray crystallography (Reinemer *et al.*, 1991; 1992; Bello *et al.*, 1992; Dirr *et al.*, 1994; Garia-Saez *et al.*, 1994; Oakley *et al.*, 1997), performed with class Pi GSTs, have contributed to our

* To whom correspondence should be addressed.
Tel: 82-2-820-5205; Fax: 82-2-825-4736
E-mail: khkong@cau.ac.kr

present understanding of the catalytic mechanism and structure of GST. However, there is little information available concerning the precise enzyme-substrate interactions that may be responsible for the catalytic properties of GST, and the identification or specific role of individual residues remains largely unknown. Also, it is unclear at present whether any other regions adjacent to a GSH-binding site (G-site) serve as a nonspecific hydrophobic site (H-site).

Therefore, in this study, the six mutants of residues in forming the GSH-binding cleft were extensively investigated with respect to the effect on the substrate specificity and the response to inhibition. This study would offer information on the chemical and structural features of the active site that dictate the substrate specificity of the enzyme and the binding sites for the nonsubstrate ligands different from the catalytic site, and provide further insight into enzyme activity and the design of enzymes.

Materials and Methods

Materials Wild-type hGST P1-1 was obtained by expression of a cloned cDNA (Kano *et al.*, 1987) in *E. coli* as described in the previous paper (Kong, *et al.*, 1991). GSH and 1,2-dichloro-4-nitrobenzene were purchased from Kohjin Co. and Wako Pure Chem. Ind. (Osaka, Japan), respectively. Cumene hydroperoxide, 1,2-epoxy-3-(*p*-nitrophenoxy) propane, and glutathione-agarose were obtained from Sigma (St. Louis, USA). Benastatin A (Aoyagi *et al.*, 1992) was a gift from Prof. Takaaki Aoyagi.

Preparation of mutant enzymes Synthesis of the oligonucleotide, site-directed mutagenesis, confirmation of mutation, construction of the expression plasmid, and expression and purification of the mutant enzymes were performed as described in the previous paper (Kong *et al.*, 1992a). All steps were performed at 4°C. The enzymes were stored at −70°C until use.

Determination of protein concentration Protein concentration of the mutant was determined by using protein assay reagent (Bio-Rad, Richmond, USA) and the wild-type enzyme as a standard protein.

Enzyme activity The specific activities were determined by measuring the initial rates of the enzyme-catalyzed conjugation of GSH with 1,2-dichloro-4-nitrobenzene or 1,2-epoxy-3-(*p*-nitrophenoxy)propane as described by Habig and Jakoby (1981). Assays were performed in a HITACHI U-2000 double-beam spectrophotometer (Hitachi Co., Tokyo, Japan) at 30°C using cuvettes of 1 cm pathlength. Initial rates were measured for 5 min, commencing 10 s after initial mixing. The reaction was initiated by 20 μl of 50 mM 1,2-dichloro-4-nitrobenzene to 860 μl of 100 mM potassium phosphate (pH 7.5) containing, in order of addition, 100 μl of 50 mM GSH and 20 μl of the enzyme. Nonenzymatic reaction rates served as controls, and were subtracted from enzymatic rates. 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane were dissolved in ethanol. The concentration of ethanol in the reaction mixture (1 ml) was constant at 2% (v/v). GSH were dissolved in H_2O immediately before use and kept in an ice-bath to prevent oxidation. The enzymes were diluted in 20 mM potassium phosphate buffer (pH 7.0) containing 3 mM EDTA, 3 mM 2-mercaptoethanol, and 20% (v/v) glycerol to a concentration that the enzymatic reaction rate was linear with time for up to 60 s after initiation, and up to a ΔA/min of 0.15. All assays were done in the presence of 0.4% (v/v) glycerol. Conditions were: (a) 1 mM 1,2-dichloro-4-nitrobenzene, 5 mM GSH, 345 nm, and (b) 0.5 mM 1,2-epoxy-3-(*p*-nitrophenoxy) propane, 5 mM GSH, 360 nm. A unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmole of product per min under the conditions of the specific assay. Specific activity is defined as the units of enzyme activity per mg of protein. GSH-dependent peroxidase activity was assayed as described (Flohe and Gnzler, 1985).

Inhibition studies The inhibitory effects on the activity of the enzyme were measured by preincubating the enzyme with 1 mM GSH and the inhibitor for 2 min, and initiating the reaction by addition of 20 μl of 50 mM 1-chloro-2,4-dinitrobenzene (final concentration, 1 mM). The concentration of inhibitor giving 50% inhibition (I_{50}) was determined from the plot of residual activity against inhibitor concentration.

Electrophoresis Denaturing SDS-PAGE was carried out by the method of Laemmli (1970) in 12.5% gels. The molecular-mass makers were SDS molecular weight standard markers (Bio-Rad) that contains phosphorylase B (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Coomassie Brilliant Blue R-250 was used for staining.

Results

Purification and PAGE of mutant enzymes The mutants of hGST P1-1 expressed in *E. coli* under the control of *tac* promoter were isolated and purified by affinity chromatography on immobilized GSH. The mutants R13T, S65A, and D98A were isolated in a yield of 1 to 3 mg/l of cultures as in the case of the wild-type. However, in the cases of K44T, Q51A, and Q64A, the amounts of the isolated enzymes were 0.1 to 0.2 mg/l of culture. As compared with the total activity in the crude extract of *E. coli* cell lysate, the activity recoveries for the wild-type, R13T, S65A, and D98A were more than 50%, but those for the other mutants were less than 10%. Thus, the binding abilities of R13T, S65A, and D98A to GSH-Sepharose were not so much different from that of the wild-type, but K44T, Q51A, and Q64A had low affinities for GSH-Sepharose. The purified wild-type and mutants gave a single band on SDS-PAGE with an apparent M_r of 25 kDa equivalent with that of the wild-type.

Substrate specificity The initial step in mercapturic acid formation is conjugation of the foreign compound with GSH, a reaction catalyzed by GSTs for many substrates (Mannervik, 1985). The initial stage in the formation of a

mercapturic acid from 1,2-dichloro-4-nitrobenzene is effected by replacement of the labile chlorine atom of the halogenonitrobenzene by GSH, the reaction being catalyzed by GST. 1,2-dichloro-4-nitrobenzene is suitable for the estimation of GST, because the absorption spectrum changes to a longer wavelength when this compound is conjugated with GSH (Fig. 1A). The specific activities of the mutants for GSH-[1,2-dichloro-4-nitrobenzene] conjugation reaction are shown in Table 1. The substitutions of the Ser65 and Asp98 with alanine resulted in approximately 75 to 80% decrease of the specific activities toward 1,2-dichloro-4-nitrobenzene. On the other hand, the substitutions of Agr13, Lys44, Gln51, and Gln64 with threonine and alanine showed an approximately 90–97% decrease. Among them, the substitution of Gln51 with threonine resulted in a more drastic decrease to 2.9% of the wild-type value.

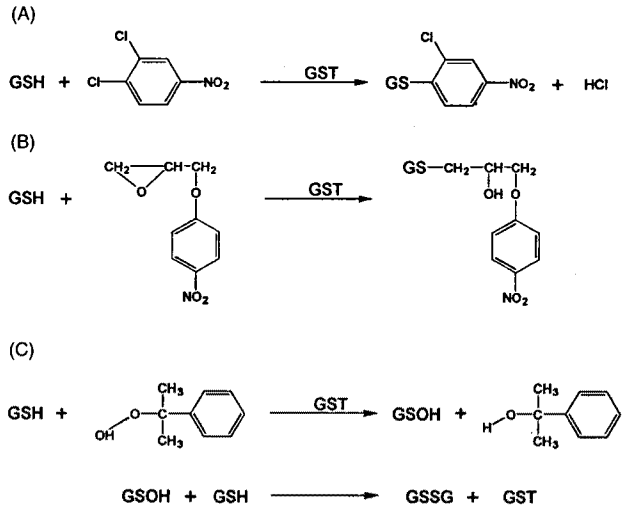

Fig. 1. The scheme of the GST-catalyzed reactions with (A) 1,2-dichloro-4-nitro-benzene, (B) 1,2-epoxy-3-(*p* nitrophenoxy)propane, and (C) cumene hydroperoxide.

Table 1. Specific activity of the wild-type and mutants for GSH conjugation with 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane.

Enzyme	1,2-dichloro-4-nitrobenzene		1,2-epoxy-3-(*p*-nitrophenoxy)propane	
	Specific activity (μmol/min/mg)	Relative activity (%)	Specific activity (μmol/min/mg)	Relative activity (%)
wild-type	0.118 ± 0.011	100.0	0.360 ± 0.038	100.0
R13T	0.014 ± 0.001	12.0	0.078 ± 0.009	21.7
K44T	0.013 ± 0.001	11.7	0.061 ± 0.005	16.9
Q51A	0.003 ± 0.001	2.9	0.035 ± 0.004	9.5
Q64A	0.009 ± 0.001	7.5	0.088 ± 0.009	24.4
S65A	0.027 ± 0.002	24.5	0.040 ± 0.004	11.1
D98A	0.023 ± 0.003	20.9	0.052 ± 0.006	14.4

Values are means ± S.D., generally based on n ≥ 5.

Epoxides are substrates that may be derivatives of naturally occurring compounds as well as of xenobiotics and are known as mutagenic and carcinogenic substances. An epoxide that has been found to be particularly useful for identifying human class Pi enzymes is 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Fig. 1B). The GSH-[1,2-epoxy-3-(*p*-nitrophenoxy)propane] conjugating activities of the mutants were assayed (Table 1). The substitutions of the active-site mutants with threonine or alanine resulted in a 75–90% decrease of the specific activities toward 1,2-epoxy-3-(*p*-nitrophenoxy)propane. The substitutions of Arg13 and Gln64 with threonine or alanine showed approximately 75% decrease. On the other hand, the substitution of Gln51 with threonine also resulted in a more drastic decrease in the specific activity. The specific activity of Q51A was less than 10% of the wild-type value.

Organic hydroperoxides are substrates for GSTs. A study involving seven cytosolic homodimeric rat transferases demonstrated that linoleate hydroperoxide and arachidonate hydroperoxide in most cases gave activities comparable to the model substrate cumene hydroperoxide (Ketterer *et al.*, 1987). The GST-catalyzed reaction with cumene hydroperoxide represents the "nonselenium" glutathione peroxidase activity and is believed to occur in two steps involving an unstable glutathione sulfonic acid intermediate (Fig. 1C). Reduced GSH is regenerated from the produced glutathione disulfide (GSSG) by the action of glutathione reductase (Mannervik, 1985). The GSH peroxidase activity of the mutants were assayed (Table 2). The substitutions of Ser65 and Asp98 with alanine resulted in approximately 81% and 73% decreases of the specific activities toward cumene hydroperoxide, respectively. On the other hand, the substitutions of Arg13, Lys44, and Gln51 with threonine or alanine resulted in further decrease. The specific activities of these mutants were less than 6% of the wild-type value.

Inhibition studies The inhibition parameters (I_{50}) of various kinds of inhibitors, benastatin A, *S*-(2,4-dinitrophenyl)glutathione, and hematin for GSH-[1-chloro-2,4-dinitrobenzene] conjugating activity

Table 2. Specific activity of the wild-type and mutants for GSH peroxidase.

Enzyme	Specific activity ($\times 10^{-2}$ μmol/min/mg)	Relative activity (%)
wild-type	3.72 ± 0.33	100.0
R13T	0.15 ± 0.03	3.9
K44T	0.22 ± 0.02	5.8
Q51A	0.20 ± 0.01	5.3
Q64A	0.31 ± 0.05	8.3
S65A	0.79 ± 0.14	21.2
D98A	1.37 ± 0.34	36.7

Values are means ± S.D., generally based on n ≥ 5.

were determined under the standard assay conditions. The substitutions of Arg13, Gln51, Gln64, Ser65, and Asp98 resulted in a small decrease in the I_{50} value of benastatin A that competes with 1-chloro-2,4-dinitrobenzene as an electrophilic substrate-like compound (Fig. 2). On the other hand, the I_{50} value for K44T was significantly higher than the wild-type value (about 4.4-fold).

The I_{50} values of *S*-(2,4-dinitrophenyl)glutathione, a conjugation product of GSH with 1-chloro-2,4-dinitrobenzene, for Q64A, S65A, and D98A were similar to the wild-type value, as shown in Fig. 3. The I_{50} values for K44T and Q51A were a little higher than the wild-type value. On the other hand, the I_{50} value for R13T was significantly lower than the wild-type value (about one-half fold).

The I_{50} values of hematin, a nonsubstrate ligand, for R13T and Q51A were significantly higher than the wild-type value (about 3- to 4-fold), as shown in Fig. 4. On the other hand, the I_{50} value for S65A was significantly lower than the wild-type value (about 3-fold). The I_{50} values for K44T, Q64A, and D98A were almost equivalent to the wild-type value.

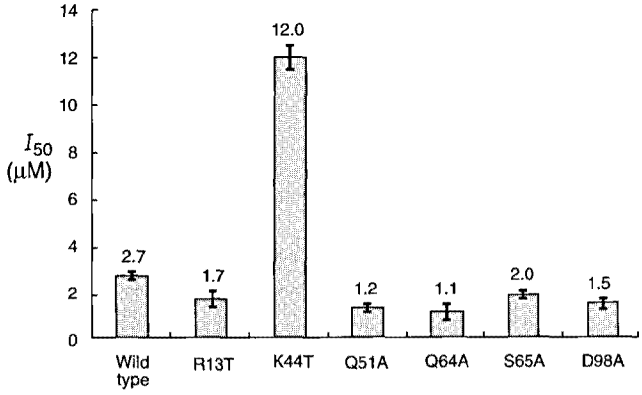

Fig. 2. Inhibition effect of the wild-type and mutants on GSH-[1-chloro-2,4-dinitrobenzene] conjugation with benastatin A.

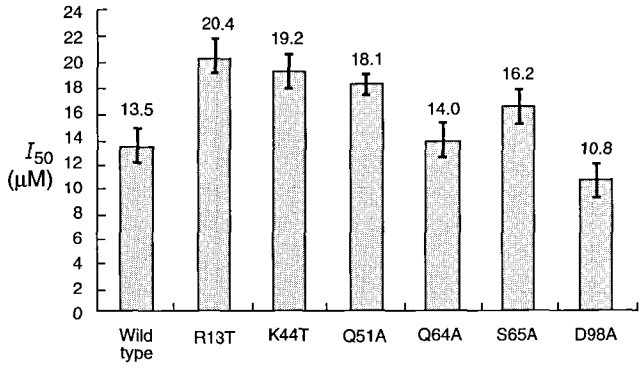

Fig. 3. Inhibition effect of the wild-type and mutants on GSH-[1-chloro-2,4-dinitrobenzene] conjugation with *S*-(2,4-dinitrophenyl)glutathione.

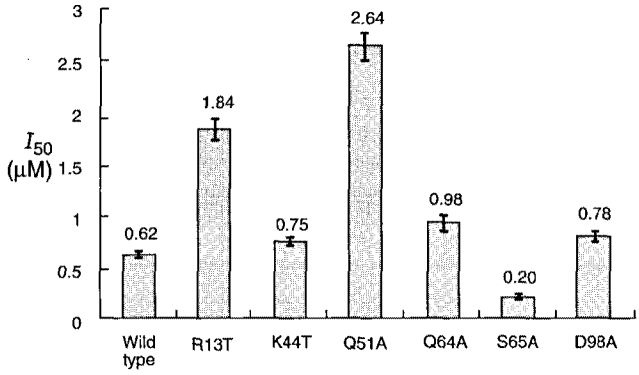

Fig. 4. Inhibition effect of the wild-type and mutants on GSH-[1-chloro-2,4-dinitrobenzene] with hematin.

Discussion

Human GST P1-1 has been extensively studied because of the clinical interest in it as a marker during chemical carcinogenesis and its potential role in the mechanism of cellular multidrug resistance against a number of antineoplastic agents (Tsuchida and Sato, 1992). Studies on the 3D-structures of porcine and human GST P1-1 suggested that Arg13, Lys44, Gln51, Gln64, Ser65, and Asp98 are located in the G-site (Reinemer *et al.*, 1991; 1992). The importance of these residues for the binding of GSH were confirmed by site-directed mutagenesis studies (Kong *et al.*, 1992a; 1993; 1994). In order to gain further insight on the relationship between structure and function of GST, the mutants of these residues were expressed in *E. coli* and purified to electrophoretic homogeneity by affinity column chromatography.

The substitutions of these mutants lowered the specific activities with 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane for GSH-conjugation reaction and with cumene hydroperoxide for GSH peroxidase activity to 3 to 25% of the values for the wild-type (Table 1 and 2). Among them, the substitution of Gln51 with threonine resulted in a more drastic decrease in the specific activities. The active-site mutants showed marked differences in the specificity on the substrates used in this study. R13T and Q64A, with about 22% and 24% of the wild-type value for GSH conjugation activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane, showed only 4% and 8% for GSH peroxidase activity with cumene hydroperoxide, respectively. On the other hand, D98A with about 14% of the wild-type value for GSH conjugation activity with 1,2-epoxy-3-(*p*-nitrophenoxy)propane showed 37% for GSH peroxidase activity with cumene hydroperoxide. These specificity differences toward the electrophilic substrate are most likely to be correlated with structural differences between their active-sites, and the substitutions of these residues might affect the interaction between the thiol group and Tyr7 through distorting the

orientation of the GSH bound in the enzyme and/or the binding site of electrophilic substrates.

The hGST P1-1 subunit consists of the N-terminal 76 residues (domain I) and the C-terminal 127 residues (domain II) which are connected by a six residue linker (Reinemer *et al.*, 1992). Most of the residues necessary for binding of GSH occupied a site on domain I (G-site). However, little is known about the location of the binding site of electrophilic substrates (H-site). The H-site must be adjacent to the G-site, and should also permit proper orientation of the bound reactants. The substitution of Arg13 with threonine resulted in the decrease of the I_{50} values for benanstatin A, an electrophilic substrate-like compound, and *S*-(2,4-dinitrophenyl)glutathione, a conjugation product of GSH with 1-chloro-2,4-dinitrobenzene (Figs. 2 and 3). Thus, the substitution of Arg13 with threonine seems to change the conformation of the active site to increase the affinity for the product and the electrophilic substrate. Arg13 is conserved in class Pi and Alpha, but is replaced with leucine in class Mu. Reinemer *et al.* (1991) suggested three possible sites for H-site of pGST P1-1: a cavity composed of residues (Gly12, Arg13, Arg100, Tyr103, Ala104, Tyr108, Val143, Asp157, Ile161, Ile203, and Asn204) in domain II, a site between the two subunits, or a hydrophobic pocket composed of residues found near the middle of α-helix D. Reinemer *et al.* (1992) also suggested that the H-site of hGST P1-1 was located adjacent to the G-site, with the hexyl moiety of *S*-hexyl-GSH bound in the enzyme lying above a segment connecting strand β1 and α-helix A. Furthermore, the three-dimensional structure of the human GST P1-1 in complex with its inhibitor ethacrynic acid implicated that the inhibitor sits in a hydrophobic pocket lined with the side chains of Tyr7, Phe8, Pro9, and Val10 and the aliphatic portions of Arg13, Val35, Ile104, and Tyr108, and the carboxylic acid moiety of the inhibitor forms a hydrogen bond to the N_ε atom of Arg13 (Oakley *et al.*, 1997). The I_{50} value of benanstatin A that competes with 1-chloro-2,4-dinitrobenzene for K44T was significantly higher than the wild-type value (approximately 4.4-fold) (Fig. 2). The substitution of this residue also resulted in a little increase in K_m values for 1-chloro-2,4-dinitrobenzene (Kong *et al.*, 1992a).

GST is known to function as ligandin that binds nonsubstrate hydrophobic ligands, such as heme, bilirubin, steroids, vitamin K, etc., and hydrophobic interaction is considered to be important for the binding of these ligands. However, since many of the ligands have anionic groups such as carboxylate, electrostatic interaction between the anionic groups of the ligands, and the cationic groups of GST, such as the side chains of an arginine and lysine residues, might also be involved in the function as ligandin. The substitutions of Arg13 and Gln51 residues significantly affect the I_{50} of hematin for GSH-[1-chloro-2,4-dinitrobenzene] conjugating activity (Fig. 4). Thus, Arg13 and Gln51 residues of hGST P1-1 are considered to be important for binding of hematin. Reinemer *et al.* (1991) suggested that an arginine residue serves as an anionic recognition site for GST and there exists the "ligandin" or the "nonsubstrate" binding site at the interface between two subunits. Nishihira *et al.* (1993) suggested that the binding of hydrophobic ligand to GST P produced a remarkable conformational change of the active center of the enzyme. Taken together, although Arg13 and Gln51 residues are thought not to interact with hematin directly, the substitutions of these residues are thought to block the hematin, possibly by inducing a conformational change of the active site.

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