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Site-directed mutagenesis of the *Proteus mirabilis* glutathione transferase B1-1 G-site

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Abstract In order to investigate the roles of near N-terminus Tyr, Cys, and Ser residues in the activity of bacterial glutathione transferase (GSTB1-1) site-directed mutagenesis was used to replace the following residues: Tyr-4, Tyr-5, Ser-9, Cys-10, Ser-11, and Ser-13. The results presented here show that, unlike all other alpha, mu, pi, theta and sigma classes of glutathione transferases so far investigated, GSTB1-1 does not utilise any Tyr, Ser or Cys residue to activate glutathione. These results also suggest that the bacterial glutathione transferases may require classification into their own class.

Key words: Glutathione transferase; *Proteus mirabilis*; Site-directed mutagenesis

1. Introduction

Glutathione transferases (GSTs; EC 2.5.1.18) consist of a family of multifunctional proteins that catalyze the nucleophilic attack of the sulfhydryl group of glutathione (GSH) to a wide number of xenobiotics including carcinogens, mutagens and therapeutic drugs [1–3]. GSTs can exist as either homo- or heterodimers with each subunit possessing both the GSH and the xenobiotic binding sites [1–3]. On the basis of structural and immunological properties the multitude of GSTs so far characterized have been classified into at least five groups, i.e. alpha, mu, pi, theta and sigma [1–3]. The three-dimensional structure of at least one member of each of the five classes of GST has been determined, revealing an overall similar structural organization, i.e. two domains joined by a short linker of six or seven amino acids [3–7]. The N-terminal GSH binding domain adopts an α/β conformation, whereas the C-terminal domain, which provides a great deal of the xenobiotic binding site, adopt an all α-helical structure [3–7].

Spectroscopic and kinetic evidence indicates that GSH is in the thiolate form (GS^-) at neutral pH when complexed with GST [8,9]. Thus, GSTs operate in catalysis by lowering the pK_a of the thiol group of the GSH molecule when it is bound to the enzyme [10]. A conserved near N-terminal tyrosine residue in alpha (Tyr-8), mu (Tyr-6), pi (Tyr-7) and sigma (Tyr-7) class GSTs and a serine residue (Ser-9) in the theta class GST are involved in the stabilization of GS^- through hydrogen bonding [3,10]. In fact, replacement of these residues with other amino acids by site-directed mutagenesis drastically reduces GST activity towards different model substrates [11–15]. On the basis of this essential of amino acid residues, involved in the catalytic activity, the various GSTs have also been classified on the basis of whether they have the critical tyrosine or serine residue [3].

In our laboratory, a GST isoenzyme, named GSTB1-1, was isolated and characterized from the Gram-negative bacterium *Proteus mirabilis* [16,17]. The sequence of GSTB1-1 has been first resolved at protein level [17], but the corresponding gene sequence is now available [18]. GSTB1-1 exhibits low sequence similarity with the GST of alpha, mu, pi, theta and sigma classes [17]. Although preliminary crystallographic data on GSTB1-1 have been published, no information on the amino acid residues involved in the catalytic mechanism is yet available [19]. An intriguing feature of GSTB1-1 is that both candidate catalytic residues that might be involved in lowering the pK_a of GSH, i.e. Tyr-5 (the bacterial counterpart of alpha, mu, pi and sigma Tyr) and Ser-9 (the bacterial counterpart of the theta Ser) are present. Therefore, it is of great interest to determine which one of the two residues present in GSTB1-1 is important for catalysis. The results of the present investigation demonstrate that neither residue is important in the catalytic mechanism of GSTB1-1.

2. Materials and methods

2.1. Chemicals

Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), as well as antibiotics used in the present work were purchased from Sigma-Aldrich (Milan, Italy). All other reagents used were of the highest grade commercially available.

2.2. Oligonucleotide-directed mutagenesis

The DNA encoding GSTB1-1 in pBtac1 (pGPT1) [18] was used as a template in the mutagenesis procedure. The single mutations Y4A, Y4S, Y5A,Y5S, Y5F, S9A, S9T, S9Y, C10A, C10S, S11A, S11T, S11Y, S13A and S13T were made with the following oligonucleotides: Y4A, 5′-T ACC CGG CGT ATA GGC CAG TTT CAT G-3′; Y4S, 5′-CT ACC CGG CGT ATA GGA CAG TTT CAT G-3′; Y5A, 5′-T ACC CGG CGT AGC GTA CAG TTT CAT G-3′; Y5S, 5′-CT ACC CGG CGT AGA GTA CAG TTT CAT G-3′, Y5F, 5′-CT ACC CGG CGT AAA GTA CAG TTT CAT G-3′; S9A, 5′-GA AAG CGA GCA AGC ACC CGG CGT ATA G-3′; S9T, 5′-GA AAG CGA GCA AGT ACC CGG CGT ATA G-3′; S9Y, 5′-GA AAG CGA GCA ATA ACC CGG CGT ATA G-3′, C10A, 5′-GG AGA AAG CGA GGC ACT ACC CGG CG-3′; C10S, 5′-GG AGA AAG CGA GGA ACT ACC CGG CG-3′; S11A, 5′-TG AGG AGA AAG CGC GCA ACT ACC CGG C-3′; S11T, 5′-TG AGG AGA AAG CGT GCA ACT ACC CGG C-3′; S13A, 5′-GAT ATG AGG AGC AAG CGA GCA ACT ACC-3′; S13T, 5′-GAT ATG AGG AGT AAG CGA GCA ACT ACC C-3′. The oligonucleotide-directed USE mutagenesis kit (Pharmacia) was used according to the manufacturer's instructions. Clones with the required mutation were first identified by colony hybridization, using the 5′-^{35}P-labelled mutameric

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oligonucleotides as probes, and confirmed by dideoxynucleotide sequencing [20].

2.3. Expression and purification of mutant GSTB1-1 enzymes

The mutant pGPT1 DNAs were used to express GSTB1-1 in *Escherichia coli* XL1-Blue as previously reported [18]. The purification of mutant enzymes was performed as reported by Di Ilio et al. [16]. Briefly, the bacterial cells were collected by centrifugation, washed twice and resuspended in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA (buffer A) and disrupted by cold sonication. The particulate material was removed by centrifugation and the resulting supernatants were applied to a GSH-Sepharose affinity column [21]. The column was exhaustively washed with buffer A, supplemented with 50 mM KCl. The enzyme was eluted with Tris-HCl buffer pH 9.6 containing 10 mM GSH. The fractions containing GST activity were pooled, concentrated by ultrafiltration, dialysed against buffer A and subjected to further analyses.

SDS/PAGE in discontinuous slab gel was performed by the method of Laemmli [22]. Protein concentration was determined by the method of Bradford [23] with γ-globulin as standard.

2.4. Kinetic studies

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was assayed at 30°C according to the method of Habig and Jakoby [24].

For the enzyme kinetic determinations either CDNB or GSH was held constant at 1 mM and 5 mM respectively, while the concentration of the other substrate was varied (from 0.1 mM to 5 mM for GSH and from 0.1 mM to 1.6 mM for CDNB). Each initial velocity was measured at least in triplicate. The data fitted well to the equation for a rectangular hyperbola. Fitting was carried out using the computer program ENZFITTER based on an iterative Gauss-Newton procedure [25].

The dependence of K_{cat}/K_m on pH was determined by using the following buffers (0.1 M) at the indicated pH: Bis-Tris-HCl, from 5.0 to 7.0; Tris-HCl, from 7.2 to 9.0. The reaction was carried out using saturating GSH (5 mM) and variable CDNB concentrations.

The pK_a values were obtained by computer fitting the data to the equation $\log(K_{cat}/K_m) = \log [C/(1+[H^+]/K_a)]$, where C is the upper limit of K_{cat}/K_m at high pH.

3. Results and discussion

Previous studies have suggested that the catalytic mechanism of alpha, mu, pi, sigma and theta class GSTs depends on their ability to lower the pK_a of the sulfhydryl group of GSH [8–10]. The resulting thiolate may act as a nucleophile to attack the electrophilic centers of a large number of xenobiotic or endobiotic molecules that bind to the enzyme [8,9]. Analyses of the three-dimensional structures of GSTs have suggested this activation role may be due to a tyrosine residue near the N-terminus in alpha, mu, pi and sigma classes, and a serine residue near the N-terminus of the theta class GST [3,10]. These suggestions have been confirmed by site-directed mutagenesis [11–15].

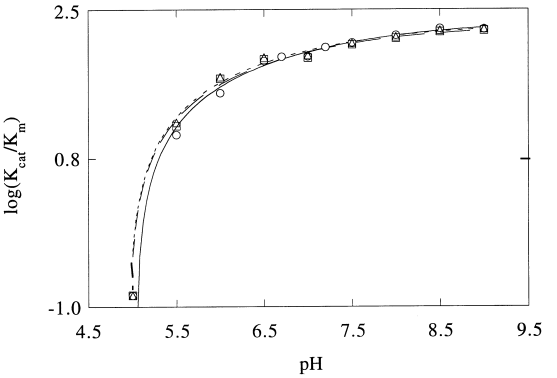

Fig. 1. pH dependence of K_{cat}/K_m^{CDNB} for the wild type (○), and the S9A (□) and Y5A (△) mutants. The lines are computer fits of the experimental data to the equation $\log(K_{cat}/K_m) = \log [C/(1+[H^+]/K_a)]$, where C is the upper limit of K_{cat}/K_m at high pH. The pK_a values derived from the computer fits are 6.4 ± 0.27, 6.3 ± 0.18 and 6.3 ± 0.37 for GSH bound to the wild type and the S9A and Y5A mutants respectively.

The GST purified in our laboratory from the microorganism *Proteus mirabilis* does not appear to be closely related to any of the GSTs from the alpha, mu, pi, sigma and theta classes [16–18]. In fact, the sequence identity between GSTB1-1 and the GST of the other classes is in the 20–25% range [17]. However, despite their low pairwise sequence identity, the N-terminal domain of GSTB1-1 possesses both a tyrosine (residue 5) and a serine (residue 9), which appear equivalent to the Tyr and Ser residues of alpha, mu, pi, sigma and theta class GSTs [17]. In order to assess the importance of these two residues in the catalytic mechanism of bacterial GST, site-directed mutagenesis experiments were carried out. The results are presented in Table 1. Both GSTB1-1 Y5A and GSTB1-1 Y5F mutants retain 70–80% of conjugating activity. On the other hand, the replacement of Ser-9 with alanine or tyrosine resulted in a 25% and 80% decrease of activity respectively. Surprisingly, the substitution of Ser-9 with threo-

Table 1
Specific activity and kinetic constants for GSTB1-1 and mutant enzymes

Enzyme	Specific activity	GSH			CDNB		
	(μmol/min/mg)	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1} s^{-1})	K_m (μM)	K_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1} s^{-1})
GSTB1-1	1.1 ± 0.08	686 ± 80	58.1 ± 6	84	730 ± 75	69.3 ± 6	95
S9A	0.8 ± 0.09	542 ± 95	44 ± 8	81	903 ± 91	79 ± 14	88
S9T	2.6 ± 0.3	586 ± 41	496 ± 27	846	708 ± 54	237 ± 22	335
S9Y	0.2 ± 0.06	658 ± 69	16 ± 5	25	744 ± 43	58 ± 4	78
S11A	0.72 ± 0.1	641 ± 15	46 ± 5	72	519 ± 35	69 ± 6	133
S11T	0.87 ± 0.2	843 ± 54	65 ± 8	77	684 ± 31	90 ± 10	132
S13A	1.07 ± 0.07	725 ± 62	58.7 ± 3	81	778 ± 47	68 ± 7	87
S13T	0.48 ± 0.18	782 ± 87	46.2 ± 8	53	841 ± 28	63 ± 9	75
C10S	0.6 ± 0.12	802 ± 37	53 ± 4	66	717 ± 18	49.5 ± 3	69
C10A	0.98 ± 0.08	784 ± 48	58.8 ± 4	75	653 ± 42	59.6 ± 6	91
Y4A	1.2 ± 0.06	799 ± 20	91.4 ± 9	73	695 ± 54	64.6 ± 8	93
Y4S	1.1 ± 0.11	884 ± 32	61 ± 11	69	632 ± 19	62 ± 5	98
Y5A	0.61 ± 0.03	876 ± 43	52.6 ± 6	60	845 ± 37	67 ± 8	72
Y5S	0.55 ± 0.1	891 ± 83	56 ± 5	63	893 ± 50	59 ± 6	66
Y5F	0.69 ± 0.1	945 ± 114	51.8 ± 12	55	910 ± 46	82 ± 7	90

The values are given as means ± S.D. as determined from replicates ($n = 3$).

nine increased the activity of the enzyme about 2.5-fold and the catalytic efficiency (K_{cat}/K_m) for GSH about 10-fold. The catalytic efficiencies towards GSH and CDNB for the Y5A, Y5F, Y5S, S9A and S9Y mutants were quite similar to that of the wild type. These results are clearly different from those obtained with the GST of alpha, mu, pi, sigma and theta classes. The substitution of the key Tyr residue in alpha, mu, pi and sigma class GSTs (equivalent to Tyr-5 in GSTB1-1), or the key Ser residue in theta class GST (equivalent to Ser-9 in GSTB1-1) leads to dramatic decreases in catalytic activity [11–15]. Thus, unlike all other GST so far studied, neither Tyr-5 nor Ser-9 of GSTB1-1 appears to be crucial in activating the enzyme-bound GSH to form the reactive glutathione thiolate anion form (GS^-). This conclusion is also supported by the results presented in Fig. 1. When the pH dependence of K_{cat}/K_m^{CDNB} was examined for the wild-type enzyme and for the Y5A and S9A mutants, no shift of the apparent pK_a of bound GSH occurred. Similar pK_a values for the wild-type enzyme (6.4 ± 0.27) and the Y5A (6.3 ± 0.37) and S9A (6.3 ± 0.18) mutants were obtained. In contrast, the replacement of the near N-terminal Tyr or Ser residues in the GST of other classes increased the apparent pK_a value of bound GSH by 1–2 pH units [3,10–14].

Since there are additional residues near the N-terminus of GSTB1-1 which are possible candidates for GSH activation (i.e. Tyr-4, Ser-11, Ser-13 and Cys-10), it was logical to ask whether one of these residues may be essential for the catalytic activity of the enzyme. Therefore the following additional GSTB1-1 mutants were prepared and investigated: Y4A, Y4S, C10A, C10S, S11A, S11T, S13A, S13T. All mutants retained more than 80% of catalytic activity and the kinetic parameters of these mutants were found to be not very different from those of the wild-type enzyme (Table 1).

In summary, the results presented here show that GSTB1-1 does not utilize any Tyr, Ser or Cys residue near the N-terminus to activate GSH. In conclusion, our results indicate that the GSTB1-1 enzyme is catalytically different from the GSTs of alpha, mu, pi, sigma and theta classes and suggest that the bacterial GSTs may require classification into their own class. It should be noted that the substitution of Ser-9 with threonine led to an increase of the catalytic efficiency of GSTB1-1, whereas the substitution of Ser-9 with the more bulky residue phenylalanine led to a decrease in the catalytic efficiency of the enzyme by about three-fold. These changes in catalytic parameters did not occur when either Ser-11 or Ser-13 was replaced by threonine. These data suggest Ser-9 may be implicated in the orientation of the sulfhydryl group of GSH in the catalytic step or in the transition state stabilization.

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