Volume 293, number 1,2, 153–155 FEBS 10409 November 1991

Mutation of an evolutionarily conserved tyrosine residue in the active site of a human class Alpha glutathione transferase

Gun Stenberg[1], Philip G. Board[2] and Bengt Mannervik[1]

[1]*Department of Biochemistry, Uppsala University, Biomedical Center, Box 576, S-751 23 Uppsala, Sweden* and [2]*Molecular Genetics Group, John Curtin School of Medical Research, Australian National University, Canberra, ACT 2061, Australia*

Received 16 September 1991

Human class Alpha glutathione transferase (GST) A1–1 has been subjected to site-directed mutagenesis of a Tyr residue conserved in all classes of cytosolic GSTs. The change of Tyr^8→Phe lowers the specific activities with three substrates to 2–8% of the values for the wild-type enzyme. The changes in the kinetic parameters k_{cat}/K_M, V_{max} and $S_{0.5}$ show that the decreased activities are partly due to a reduced affinity for glutathione. The effect is reflected in lowered k_{cat} values, suggesting that the hydroxyl group of Tyr^8 is involved in the activation of glutathione. The proposal of such a role for the Tyr residue has support from the 3D structure of a pig lung class Pi GST [Reinemer et al. (1991) EMBO J. 10, 1997–2005]. Thus, Tyr^8 appears to be the first active site residue established as participating in the chemical mechanism of a GST.

Glutathione transferase; Human; Site-directed mutagenesis; Active-site tyrosine

1. INTRODUCTION

Comparison of primary structures of homologous proteins is one approach that can be used to establish amino acid residues of significance for the structure and function of protein molecules. In the cytosolic glutathione transferases characterized, about 5% of the approximately 220 amino acid residues are conserved in all the major classes of the enzymes [1]. Some of these are arginine residues and a recent investigation based on site-directed mutagenesis indicated that such residues are possibly involved in binding of the cofactor glutathione [2]. A tyrosine residue near the N-terminus is also highly conserved [1], and has been targeted for mutagenesis experiments. In the work described here this residue (Tyr^8) has been mutated into Phe in human class Alpha glutathione transferase A1–1*. The relevance of this mutation became accentuated when an X-ray diffraction investigation demonstrated that the corresponding tyrosine residue in a pig lung class Pi enzyme is positioned close to a glutathione analogue in the active site [3]. The results of the present investigation strongly suggest that Tyr^8 contributes to enzyme catalysis, even though it is not essential for catalysis to occur.

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; GSH, glutathione

Correspondence address: Bengt Mannervik, Department of Biochemistry, Uppsala University, Biomedical Center, Box 576, S-751 23 Uppsala, Sweden. Fax: (46) (18) 558 431.

*The enzyme denoted as human class Alpha glutathione transferase A1-1 has also been named GST2 type 1, GST_2 and $GSTB_1B_1$

2. MATERIALS AND METHODS

2.1. *Chemicals*

Sephadex G-25 and epoxy-activated Sepharose 6B were from Pharmacia LKB Biotechnology, Uppsala, Sweden. *S*-Hexylglutathione and *S*-hexylglutathione Sepharose 6B were synthesized as previously described [4]. Substrates for enzyme activity measurements and other chemicals were of highest quality available from commercial sources. Δ^5-Androstene-3,17-dione was generously provided by Dr. Paul Talalay (Johns Hopkins University, Baltimore, MD, USA). Oligonucleotide-directed in vitro mutagenesis kit and [^{32}P]dCTP were from Amersham International. Restriction enzymes, and other DNA-modifying enzymes as well as plasmids were obtained from Amersham International (Promega Corp., Boehringer Mannheim and Pharmacia LKB Biotechnology). Mutagenic oligonucleotides were synthesized at the Department of Immunology, Uppsala University, Uppsala, Sweden.

2.2. *Enzymes*

Plasmid pTacGST2 [5] containing the cDNA for the wild-type enzyme A1–1 was used for expression of the protein. After subcloning of the coding sequence in M13mp18, oligonucleotide-directed mutagenesis [6] was performed to create a Tyr^8→Phe change in the structure by methods previously described [2]. Wild-type and mutant enzymes were expressed in *E. coli* JM103, and purified by affinity chromatography on an *S*-hexylglutathione-matrix [4]. Purity of the enzyme preparations were checked by HPLC on an C4 column (cf. [7]) and by SDS/PAGE [8].

2.3. *Assays of enzyme activity*

Specific activities of the purified enzymes were determined at 30°C in three assay systems. Conjugation between GSH (1 mM) and CDNB (1 mM) was monitored spectrophotometrically at 340 nm in 0.1 M sodium phosphate (pH 6.5) containing 1 mM EDTA (v/v)/5% EtOH (solvent for CDNB) [9]. Peroxidase activity was measured at 340 nm in a system containing 1.5 mM CuOOH, 1 mM GSH, 0.3 units of glutathione reductase, and 0.1 mM NADPH, 1 mM EDTA and 5% EtOH (solvent for CuOOH) in 0.1 M sodium phosphate (pH 7.0) [10]. Isomerase activity was monitored at 248 nm in a reaction system containing 0.1 mM GSH, 0.1 mM dithiothreitol and 0.068 mM Δ^5-androstene-3,17-dione, in 0.025 M Tris/0.0125 M potassium-phosphate (pH 8.5), and 2% MeOH (solvent for steroid) [11].

The effect of inhibitors on catalytic activity was determined in the

Table I

Specific activities of human glutathione transferase A1–1 mutant Y8F

Substrate	Specific activity (μmol·min^{-1}·mg^{-1})	Percent of wild-type
1-Chloro-2,4-dinitrobenzene	1.9	3
Cumene hydroperoxide	0.8	8
Androstenedione	0.2	2

assay system involving CDNB as electrophilic substrate. The inhibitor was added to the ongoing reaction 2 min from start. I_{50} values were calculated from plots of remaining activity vs. inhibitor concentration [12].

3. RESULTS AND DISCUSSION

Oligonucleotide-directed mutagenesis of the cDNA encoding human glutathione transferase A1–1 was performed as previously described [2] using the mutamer 5'-GCA TTG AAG AAG TGG AGC TT-3' in order to change codon 8, TAC (Tyr), into TTC (Phe). The resulting mutant cDNA was sequenced [13] in its entirety and it was verified that no alterations of the sequence other than the desired mutation had occurred.

The protein corresponding to the mutated cDNA encoding human glutathione transferase A1–1 was expressed in *Escherichia coli* strain JM103. Purification of the recombinant protein was based on affinity chromatography on *S*-hexylglutathione [4] and was carried out as previously described [2]. A final yield of 8 mg was obtained from a 3 liter culture. Judging from the results of the purification, the affinity for the *S*-hexyl glutathione matrix was not altered noticeably by the mutation, suggesting that the binding of the ligand was largely unimpaired. Physical properties such as electrophoretic mobility and apparent M_r were indistinguishable from those of the wild-type enzyme. The N-terminal amino acid sequence was determined on an Applied Biosystems 477A gas phase amino acid sequencer. Like the wild-type recombinant enzyme A1–1 [2], the mutant protein contained Ala at the N-terminus, which corresponds to amino acid residue no. 2 in the deduced amino acid sequence. The only difference detected in the segment analyzed (15 residues) was that position no. 8 contained Phe in the mutant protein rather than Tyr in the wild-type structure. This result confirms the mutation of the DNA structure at the protein level.

The specific activity of the mutant enzyme was tested with three substrates, 1-chloro-2,4-dinitrobenzene, cumene hydroperoxide and androstenedione, known to give the highest values for the wild-type enzyme (cf. [11]). The mutant enzyme gave low but measurable activities ranging between 2% and 8% of the corresponding values of the wild-type enzyme (Table I). The enzyme does not obey Michaelis-Menten kinetics, but k_{cat}/K_M values were estimated from the slope of the V vs. [S] curve (cf. [14]). Like the specific activities, the k_{cat}/K_M values ranged between 2% and 5% of the values for the wild-type enzyme. V_{max} and $S_{0.5}$ were also estimated from the rate saturation curve. Even if the data are difficult to interpret in mechanistic terms, owing to the complexity of the kinetics [1], it is clear that the low specific activity was due primarily to an effect on the turnover number; V_{max} values were 4–9% of the wild-type values, whereas the $S_{0.5}$ values were similar to the references values (Table II). The rate limiting step(s) in the catalytic mechanism is unknown and, in general, the k_{cat} and V_{max} values could be linked to different steps in the catalytic mechanism such as formation of chemical bonds between reactants, isomerization of enzyme–substrate complexes, and dissociation of a product from the enzyme. However, the results of the present investigation demonstrate that the apparent affinity (I_{50}) for the glutathione derivatives, which are product analogues, is not altered markedly by the Tyr^8→Phe substitution, suggesting that the mutation affects primarily chemical steps in the catalytic mechanism rather than dissociation of product. The clear increase of the $S_{0.5}$ value for glutathione (Table II) is consistent with the X-ray analysis [3] suggesting an interaction between the hydroxyl group of Tyr^8 with the glutathione molecule. The small but unambiguous effect on the binding (I_{50}) of glutathione derivatives, detectable only by lowering the glutathione concentration owing to competition, supports

Table II

Kinetic parameters for human glutathione transferase A1–1 mutant Y8F

Varied substrate	k_{cat}/K_m (mM^{-1}·s^{-1})		V_{max} (μM·min^{-1}·mg^{-1})		$S_{0.5}$ (mM)	
		(% wild-type)		(% wild-type)	Y8F	wild-type
GSH	5.8	(2)	2.9	(5)	0.5	0.1
CDNB	4.8	(5)	2.8	(4)	0.5	0.5
CuOOH	1.0	(3)	1.2	(9)	0.5	0.3

The $k_{cat}/_m$ values were derived from measurements at low substrate concentrations where the rate-saturation curve degenerates to first-order with respect to the concentration of the varied substrate (cf. [14]): glutathione (GSH) ≤ 50 μM ([CDNB] fixed at 1 mM); 1-chloro-2,4-dinitrobenzene (CDNB) ≤ 50 μM ([GSH] fixed at 1 mM); cumene hydroperoxide (CuOOH) ≤ 120 μM ([GSH] fixed at 1 mM). V_{max} and $S_{0.5}$ ([S] giving ½ V_{max}) values were estimated from the complete rate-saturation curves. Values for the wild-type enzyme [2] are included for comparison.

Table III

Inhibition characteristics of human glutathione transferase A1–1 mutant Y8F

Inhibitor	[glutathione] (mM)	I_{50} (μM)	
		mutant Y8F	wild-type
S-Hexylglutathione	1.0	0.9	0.7
S-Hexylglutathione	0.2	0.4	0.2
S-Methylglutathione	1.0	3000	3000
S-Methylglutathione	0.2	1800	500
Bromosulfophthalein	1.0	6	5

I_{50} values were determined as the inhibitor concentration giving 50% inhibition at constant glutathione (as indicated) and 1-chloro-2,4-dinitrobenzene (1 mM) concentrations (cf. [12])

this interpretation. The inhibition caused by bromosulfophthalein, which is believed to bind at a separate site on the enzyme [15], is not affected to any measurable extent (Table III).

The decision to make the Tyr^8→Phe mutation described in the present study was based on the observation that Tyr^8 is conserved in the cytosolic glutathione transferases. The hydropathy plots of the cytosolic glutathione transferases of classes Alpha, Mu and Pi [16] strongly indicate that the chainfold is similar for all of the enzymes, even if the identities in amino acid residues are limited to approximately 30% between members of the different classes. Therefore, the interpretation of the results of the Tyr^8→Phe mutation can now be made in the light of information obtained from the three-dimensional structure of the pig lung class Pi glutathione transferase [3], since it appears obvious that Tyr^8 is located in the active site of human glutathione transferase A1–1 in a similar manner as the conserved Tyr in the class Pi structure. The results obtained in this study indicate that the conserved Tyr^8 residue serves an important role in the active site of all glutathione transferases. Assuming that glutathione binds to the active site of the human class Alpha enzyme in a similar manner as the glutathione sulfonate molecule binds in the active site of the pig lung transferase [3], the data presented in Tables II and III are consistent with the interpretation that the hydroxyl group of Tyr^8 is involved in the activation of the sulfhydryl group of the glutathione molecule, probably by formation of a hydrogen bond. In this manner the enzyme may facilitate ionization and promote the formation of the thiolate nucleophile [17]; Tyr^8 could serve as a hydrogen bond acceptor or as a base. It is not clear how this would affect the reaction catalyzed by androstenedione (Table I), since the role of glutathione in the reaction is probably only indirect [18]. However, it is noteworthy that steroid isomerase from *Pseudomonas testosteroni*, acting on the same substrate, also has a Tyr residue participating in the catalytic mechanism [19]. However, in steroid isomerase the role of the Tyr residue is to serve as an acid rather than as an acceptor of a proton or of a hydrogen bond. The detailed understanding of the chemical mechanism of catalysis of the glutathione transferases requires further studies of the relationship between structure and function. Nevertheless, the demonstration that the hydroxyl group of Tyr^8 critically affects the k_{cat} (or V_{max}) value, establishes Tyr as the first amino acid residue identified as involved in the catalysis.

Acknowledgements: We thank Dr. Paul Talalay, Johns Hopkins University, School of Medicine, Baltimore, MD, USA for generously supplying Δ^5-androstene-3,17-dione for our experiments. Ms. Eva Holmström has given valuable assistance in the purification of the enzyme. This work was supported by the Swedish Natural Science Research Council, the Swedish Council for Engineering Sciences, and the Carl Trygger Foundation.

REFERENCES

[1] Mannervik, B. and Danielson, U.H. (1988) CRC Crit. Rev. Biochem. 23, 283–337.
[2] Stenberg, G., Board, P.G., Carlberg, I. and Mannervik, B. (1991) Biochem. J. 274, 549–555.
[3] Reinemer, P., Dirr, H.W., Ladenstein, R., Schäffer, J., Gallay, O. and Huber, R. (1991) EMBO J. 10, 1997–2005.
[4] Mannervik, B. and Guthenberg, C. (1981) Methods Enzymol. 77, 231–235.
[5] Board, P.G. and Pierce, K. (1987) Biochem. J. 248, 937–941.
[6] Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8765–8785.
[7] Ostlund Farrants, A.-K., Meyer, D.J., Coles, B., Southan, C., Aitken, A., Johnson, P.J. and Ketterer, B. (1987) Biochem. J. 245, 423–428.
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Habig, W.H. and Jakoby, W.B. (1981) Methods Enzymol. 77, 398–405.
[10] Lawrence, R.A. and Burk, R.F. (1976) Biochem. Biophys. Res. Commun. 71, 952–958.
[11] Benson, A.M., Talalay, P., Keen, J.H. and Jakoby, W.B. (1977) Proc. Natl. Acad. Sci. USA 74, 158–162.
[12] Tahir, M.K. and Mannervik, B. (1986) J. Biol. Chem. 261, 1048–1051.
[13] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[14] Danielson, U.H. and Mannervik, B. (1985) Biochem. J. 231, 263–267.
[15] Bhargava, M.M. and Dasgupta, A. (1988) Biochim. Biophys. Acta 955, 296–300.
[16] Persson, B., Jörnvall, H., Ålin, P. and Mannervik, B. (1988) Protein Seq. Data Anal. 1, 183–186.
[17] Chen, W.-J., Graminski, G.F. and Armstrong, R.N. (1988) Biochemistry 27, 647–654.
[18] Mannervik, B. (1990) in: Biological Oxidation Systems, vol. 1 (Reddy, C.C., Hamilton, G.A. and Madyastha, K.M. eds.) pp. 515–526, Academic Press, San Diego.
[19] Kuliopulos, A., Mildvan, A.S., Shortle, D. and Talalay, P. (1989) Biochemistry 28, 149–159.