Novel class of glutathione transferases from cyanobacteria exhibit high catalytic activities towards naturally occurring isothiocyanates

Eric WIKTELIUS and Gun STENBERG[1]
Department of Biochemistry and Organic Chemistry, Biomedical Centre, Uppsala University, Box 576, SE-751 23 Uppsala, Sweden

In the present paper, we report a novel class of GSTs (glutathione transferases), called the Chi class, originating from cyanobacteria and with properties not observed previously in prokaryotic enzymes. GSTs constitute a widespread multifunctional group of proteins, of which mammalian enzymes are the best characterized. Although GSTs have their origin in prokaryotes, few bacterial representatives have been characterized in detail, and the catalytic activities and substrate specificities observed have generally been very modest. The few well-studied bacterial GSTs have largely unknown physiological functions. Genome databases reveal that cyanobacteria have an extensive arsenal of glutathione-associated proteins. We have studied two cyanobacterial GSTs which are the first examples of bacterial enzymes that are as catalytically efficient as the best mammalian enzymes. GSTs from the thermophile *Thermosynechococcus elongatus* BP-1 and from *Synechococcus elongatus* PCC 6301 were found to catalyse the conjugation of naturally occurring plant-derived isothiocyanates to glutathione at high rates. The cyanobacterial GSTs studied are smaller than previously described members of this enzyme family, but display many of the typical structural features that are characteristics of GSTs. They are also active towards several classical substrates, but at the same moderate rates that have been observed for other GSTs derived from prokaryotes. The cloning, expression and characterization of two cyanobacterial GSTs are described. The possible significance of the observed catalytic properties is discussed in the context of physiological relevance and GST evolution.

Key words: cyanobacterium, detoxification, glutathione transferase (GST), isothiocyanate, microbial enzyme.

INTRODUCTION

The enzyme family of GSTs (glutathione transferases) (EC 2.5.1.18) hosts multifunctional proteins with representatives in all oxygen-utilizing organisms representing all kingdoms. Mammalian GSTs are the best characterized enzymes and their major function is generally considered to be to act as detoxifying enzymes. Considerably less is known about prokaryotic GSTs. GSTs catalyse the conjugation of the tripeptide glutathione (GSH) (γGlu-Cys-Gly) to electrophilic centres in a wide variety of potentially harmful compounds. This results in more water-soluble conjugates that can be further metabolized and eventually excreted. Most organisms express several isoenzymes with somewhat different, but often overlapping, substrate specificities. Mammalian soluble GSTs are grouped into at least eight classes on the basis of similarities in primary structure: Alpha, Mu, Omega, Pi, Sigma, Theta and Zeta belong to the cytosolic family, whereas the Kappa class of enzymes are mitochondrial and constitute their own family. A third enzyme family comprise the membrane-associated proteins denoted MAPEG (membrane-associated proteins in eicosanoid and glutathione metabolism). In addition, there are distinct classes in insects, plants and microorganisms. All known crystallized cytosolic GSTs are dimeric proteins where each subunit is composed of two domains [1–5]. Most of the GSH-binding amino acid residues forming the G-site are located in the N-terminal domain, whereas the C-terminal part makes most contacts with the electrophilic substrate and defines the H-site [6]. Kappa class enzymes have a somewhat different topology and also the membrane-associated proteins are structurally different from the cytosolic variants [1].

Glutathione metabolism is an ancient feature in the history of life, dating back to the evolution of an oxygen-containing atmosphere [7,8]. GSH and GSTs are thought to have developed as a response to the increase in oxygen and the reactive oxygen species thus generated. Oxygen itself was introduced by the photosynthetic action of cyanobacteria. Most prokaryotes have GST genes (archaea being exceptions), but so far relatively few prokaryotic GSTs have been functionally and structurally characterized (for reviews, see [2,5,7]). Although the overall sequence similarity between GSTs is small, typically less than 30 % between classes, the structural scaffold is highly conserved between classes and even organisms. Early classifications assigned prokaryotic GSTs to the Theta class of enzymes [9], but they are now considered to be unique classes. However, most bacterial GSTs remain solely as annotations in sequenced genomes. Among bacterial GSTs that have been described, PmGST B1-1, the enzyme from *Proteus mirabilis*, denoted as a prototype for bacterial GSTs [10], and *Escherichia coli* [11], are typical representatives. They show poor catalytic activity and low specificity towards standard GST substrates such as CDNB (1-chloro-2,4-dinitrobenzene) and peroxides. In fact, bacterial GSTs have been suggested not to be primarily detoxifying enzymes, but rather to be involved in primary metabolism. As a consequence, these enzymes catalyse one particular reaction and have specificity for one substrate. During the course of evolution, the function of bacterial GSTs may have changed. It has been suggested that the

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; GST, glutathione transferase; IPTG, isopropyl β-D-thiogalactoside; ITC, isothiocyanate; NBC, 4-nitrobenzylchloride; NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; PmGST, GST from *Proteus mirabilis*; SeGST, GST from *Synechococcus elongatus* PCC 6301; TeGST, GST from *Thermosynechococcus elongatus* BP-1.

[1] To whom correspondence should be addressed (email Gun.Stenberg@biorg.uu.se).

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PmGST B1-1 have evolved from a redox enzyme to a transferase enzyme [12].

Cyanobacteria have high concentrations of GSH in their cytosol [8], indicating the presence of GSH-utilizing enzymes. Cyanobacteria may very well be the first organisms to harbour GSTs. Indeed, all known cyanobacterial genomes do contain various numbers of genes annotated as GSTs as well as other GSH-dependent proteins. The enzymes described in the present paper represent a new variant of GST that appears to be specific for cyanobacteria. The cyanobacterial GSTs are smaller than traditionally studied GSTs, and the proteins have not been investigated or described before. As judged by completely sequenced genome information, the genome of the thermophilic cyanobacterium *Thermosynechococcus elongatus* BP-1 contains one single gene with GST annotation, encoding a protein of only 186 amino acids as opposed to most cytosolic GSTs containing 200–250 amino acids. For comparison, a GST of a similarly smaller size from a frequently used laboratory strain *Synechococcus elongatus* PCC 6301 was also selected. The genome (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) reveals that this latter strain also contains other soluble GSTs more similar in size to previously studied GSTs from eukaryotes, as well as a microsomal GST.

Among the electrophilic substances processed by GSTs, the conjugation reaction between ITCs (isothiocyanates) and GSH has received particular attention [13,14]. ITCs are plant substances that are released after cleavage of glucosinolates by the plant enzyme myrosinase (a thioglucosidase), as a response to injury or other forms of stress. ITCs are part of the plant defence mechanism with antimicrobial effects and can also act as repellents or attractants for insects. Possibly the most studied aspect of ITCs is the anti-carcinogenic effect that ingestion of ITCs has on mammalian cells [13,15], largely explained by the induction of phase II detoxifying enzymes (see [16] and references therein).

The present study is the first on cyanobacterial GSTs and shows that bacterial GSTs can be as efficient catalysts as the best mammalian enzymes. Plant-derived antimicrobial compounds aimed at plant defence are excellent substrates for cyanobacterial GSTs. Ancient GSTs from when they first evolved might thus have had a role in detoxification reactions and not primarily in metabolic processes. We suggest that these cyanobacterial GSTs are placed in a new class of GST called the Chi class.

EXPERIMENTAL

Materials

T. elongatus culture and the recipe for DTN medium were kindly provided by Dr Miwa Sugiura (Department of Applied Biological Chemistry, Faculty of Agriculture, Osaka Prefecture University, Osaka, Japan). *S. elongatus* culture was obtained from the Pasteur Institute and *E. coli* XL-1 Blue from Stratagene. Chemicals were purchased either from Sigma–Aldrich or Merck, except tryptone for bacterial growth medium, which was from Oxoid. Oligonucleotides were purchased from Thermo, Pfu DNA polymerase was from Stratagene, restriction enzymes were from Roche (EcoRI) and Fermentas (HindIII), T4 DNA ligase was from Roche and dNTPs were from Amersham Biosciences.

Culturing cyanobacteria

DTN medium was prepared from seven stock solutions diluted with sterile distilled water. A 40× DTN stock solution contained 7.6 g/l bisodium EDTA, 4 g/l $MgSO_4 \cdot 7H_2O$, 4 g/l KNO_3, 28 g/l $NaNO_3$, 7 g/l $NaHPO_4 \cdot 2H_2O$, 2.8 g/l $CaCl_2 \cdot 4H_2O$ and 0.32 g/l NaCl. The sulfite/thiosulfate solution contained 40 g/l $Na_2S_2O_3 \cdot 5H_2O$, 20 g/l Na_2SO_3 and 33.6 g/l $NaHSO_3$. The micronutrients solution contained 0.5 ml/l H_2SO_4 (concentrated), 0.5 g/l H_3BO_4, 2.7 g/l $MnSO_4 \cdot H_2O$ g/l, 0.5 g/l $ZnSO_4 \cdot 7H_2O$, 0.025 g/l $CuSO_4 \cdot 5H_2O$, 0.025 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.045 g/l $CoCl_2 \cdot 6H_2O$, 0.019 g/l $(NH_4)_2Ni(SO_4)_2 \cdot 6H_2O$ and 0.004 g/l Na_2SeO_4. Other solutions prepared separately were 3.9 g/l $FeCl_3 \cdot 6H_2O$, 179.2 g/l tricine (adjusted to pH 8 with NaOH), 53.45 g/l NH_4Cl and 84 g/l $NaHCO_3$. Then, 25 ml of DTN base, 0.26 ml of $FeCl_3$, 5 ml of sulfite/thiosulfate solution, 16 ml of tricine, 0.2 ml of NH_4Cl and 0.5 ml of micronutrients solution were mixed, the pH was adjusted to 7.6 with NaOH, and the volume was adjusted to 990 ml with sterile distilled water. The medium was autoclaved, and, finally, 10 ml of 0.2 μm filter-sterilized $NaHCO_3$ solution was added. BG-11 medium contained 1.5 g/l $NaNO_3$, 0.04 g/l $K_2HPO_4 \cdot 3H_2O$, 0.075 g/l $MgSO_4 \cdot 7H_2O$, 0.032 g/l $CaCl_2 \cdot H_2O$, 0.006 g/l citric acid, 0.006 g/l ferric ammonium citrate, 0.001 g/l EDTA, 0.02 g/l Na_2CO_3 and 1 ml/l of trace metal mix [2.86 g/l H_3BO_4, 1.81 g/l $MnCl_2 \cdot 4H_2O$, 0.222 g/l $ZnSO_4 \cdot 7H_2O$, 0.39 g/l $Na_2MoO_4 \cdot 2H_2O$, 0.079 g/l $CuSO_4 \cdot 5H_2O$ and 0.049 g/l $Co(NO_3)_2 \cdot 6H_2O$]. Slant-growing *S. elongatus* cells were inoculated and grown in 20 ml of BG-11 medium for 5 days at room temperature (∼25 °C) under a conventional 75 W light bulb with occasional manual agitation until the culture was dense. A 200 μl volume of *T. elongatus* culture was added to 30 ml of DTN medium and grown at 55 °C for 10 days with slow agitation.

Cloning of GSTs from cyanobacteria

Amplification of GST-encoding genes was achieved by PCR. Oligonucleotide sequences used as primers were 5′-AAGAATTCATGCTCAAGCTCTATGGCGGCGCC-3′ and 5′-AAAAGCTTTCATGCCCTCGCTCCCATTAAACC-3′ for TeGST (glutathione transferase from *Thermosynechococcus elongatus* BP-1) based on GenBank® sequence number NC_004113 and 5′-AAGAATTCATGCTGAAGCTGTACGGCAGCGCT-3′ and 5′-AAAAGCTTCTAGCGCCGACCAATCGACTGTTG-3′ for SeGST (glutathione transferase from *Synechococcus elongatus* PCC 6301) based on GenBank® sequence number NC_006576. The underlined nucleotides in both primer pairs constitute an AA overhang and restriction sites for EcoRI and HindIII respectively. The reactions contained 4 μl (1 μl of 10-fold diluted for *T. elongatus*) of cell culture as template, 0.8 μM of each primer, 0.2 mM dNTPs and 2.5 units of Pfu DNA polymerase in a total reaction volume of 50 μl. The programme consisted of 95 °C for 10 min followed by 35 cycles of 95 °C for 1 min, 60 °C for 1 min (68 °C for 1.5 min for *T. elongatus*) and 72 °C for 1 min, after which the reaction was completed at 72 °C for 30 min. The DNA products were recovered from an agarose gel after separation. The DNA was subcloned into pGEM3-Z (Promega) and used for transformation of competent *E. coli* cells [17]. The cells were grown overnight, plasmids were prepared, and the DNA was digested with restriction enzymes. After separation by agarose gel electrophoresis, the fragment was purified once again and ligated into expression vector pKK-D [18], before transformation of *E. coli* cells. For small-scale expression tests, bacteria were cultured in 5 ml of Luria–Bertani medium at 37 °C with 0.1 mg/ml ampicillin. Expression was induced by addition of IPTG (isopropyl β-D-thiogalactoside) to a final concentration of 1 mM, and the cultures were then allowed to grow for 16 h. The cells were disrupted by ultrasonication, and the lysates were analysed by SDS/PAGE to verify overexpression. The DNA used for expression was sequenced in full to verify that no mutations had been introduced.

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Table 1 Assay conditions for substrates used to characterize GSTs

All measurements were performed at 30 °C in 0.1 M potassium phosphate buffer. Changes in absorbance at the stated wavelengths were related to the molar absorption coefficients as described in the Experimental section.

Substrate	Assay pH	Molar absorption coefficient ($M^{-1} \cdot cm^{-1}$)	Wavelength (nm)	Substrate concentration (mM)	GSH concentration (mM)
CDNB	6.5	9600	340	1	1
DCNB	6.5	10 000	344	1	5
NBC	6.5	1900	310	0.25	5
Ethacrynic acid	6.5	5000	270	0.1	1
EPNP	6.5	5000	360	0.5	5
CuOOH	7.0	−6300	340	1.5	1
H_2O_2	7.0	−6300	340	1.5	1
NBD-Cl	6.5	14 500	419	0.1	5
Trans-2-nonenal	6.5	−13 750	224	0.1	0.5
Phenethyl-ITC	6.5	8890	274	0.4	4
Allyl-ITC	6.5	7450	274	0.4	1
Propyl-ITC	6.5	8350	274	0.4	4
Cyclohexyl-ITC	6.5	8520	274	0.1	4
Sulforaphane	6.5	8000	274	0.4	1
Iodomethane	8.0	4500	226	1	1
1-Iodopenthane	8.0	4500	226	1	1
Dichloromethane	8.5	8000	412	5	1

Protein expression and purification

For large-scale expression, each expression construct was grown in six lots of 0.5 litre 2× TY medium (16 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl) supplemented with 0.1 mg/ml ampicillin in 2 litre Erlenmeyer flasks at 37 °C. Induction was performed by addition of IPTG to a concentration of 1 mM at an A_{600} of 0.45–0.55. The cells were allowed to grow for an additional 16–18 h at 37 °C (TeGST) and 30 °C (SeGST). Cells were harvested by centrifugation at 4 °C at 12 000 ***g*** for 15 min, resuspended in 10 mM Tris/HCl, pH 7.8, containing 1 mM EDTA, and approx. 500 mg of lysozyme was added. After 2 h of incubation on ice, the cells were disrupted further by ultrasonication. Lysed cells were centrifuged at 4 °C at 27 000 ***g*** for 1 h. The lysate was mixed with 25 ml of Glutathione Sepharose™ 4 Fast Flow (Amersham Biosciences). NaN_3 and PMSF were added to 0.02 % (w/v) and 50 μM respectively, and proteins bound at 4 °C overnight. The gel was washed with 10 mM Tris/HCl, pH 7.8, and 1 mM EDTA, and proteins were eluted with 50 mM glycine, pH 10. The pH of the eluted fractions was immediately adjusted with 1/12 volume of 2 M Tris/HCl, pH 7.2. The samples were passed through PD-10 Sephadex™ G-25 columns and dialysed against 15 litres of 10 mM Tris/HCl, pH 7.8, and 1 mM EDTA in three changes. Alternatively, enzymes were eluted using 50 mM GSH and 10 mM Tris/HCl, pH 7.8, and 1 mM EDTA. GSH was removed by dialysis as described above. Purified proteins were analysed by the Amino Acid Analysis Laboratory of the department to obtain the mass of the protein sample and its amino acid composition. The results obtained and the Beer–Lambert Law were used for calculation of a specific absorption coefficient at 280 nm for each protein.

Enzyme kinetics

Enzyme activities were measured for CDNB, DCNB (1,2-dichloro-4-nitrobenzene), ethacrynic acid, NBC (4-nitrobenzylchloride) and EPNP [1,2-epoxy-3-(*p*-nitrophenoxy)propane] using the methods of Habig and Jakoby [19]. Other substrates used were CuOOH (cumene hydroperoxide) and H_2O_2 [20], NBD-Cl (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) [21], *trans*-2-nonenal [22], phenethyl-ITC, allyl-ITC, propyl-ITC, cyclohexyl-ITC, sulforaphane [23], iodomethane, 1-iodopenthane [24] and dichloromethane [25]. The electrophilic substrates were dissolved in organic solvents, ethanol or acetonitrile, to final concentrations of 2–5 % (v/v) of the organic solvent in the assay solutions. The assays were conducted in 0.1 M sodium phosphate buffer at 30 °C under the conditions described in Table 1. Measurements for determination of the kinetic constants K_m, k_{cat} and k_{cat}/K_m were performed at 30 °C for GSH and phenethyl-ITC. Saturating conditions were 4 mM for GSH and 0.8 mM for phenethyl-ITC. Activities were measured at eight different concentrations of GSH and phenethyl-ITC ranging from 0.01 to 4 mM and 0.025 to 0.8 mM respectively. Kinetic constants were obtained by analysis using the GraphPad Prism software. The inhibition by Cibacron Blue and hexyl glutathione was studied using phenethyl-ITC as an electrophilic substrate under the same conditions as above. IC_{50} values were calculated as the concentration of inhibitor that gives 50 % of the reaction rate of the uninhibited reaction.

Thermal stability assay

The thermal stability of the proteins was measured by a heat inactivation assay. Three samples of equal concentration (28 μg/ml) were prepared for each temperature and incubated for 10 min. An aliquot (50 μl) of the sample was then withdrawn for activity measurements with CDNB or phenethyl-ITC as the electrophilic substrate at 30 °C as described above. Thermal stability experiments were performed for every new preparation of enzyme. Specific activity was measured after heat incubation with CDNB and/or phenethyl-ITC as substrate.

Quaternary structure determination

Molecular masses and pI values for monomers of both proteins were estimated using the ExPASy Proteomics Server (http://www.expasy.org). The possible multimeric structures of the native proteins were analysed using non-denaturing PAGE (PhastSystem™; Amersham Biosciences). To distinguish between monomeric and dimeric structures of TeGST and SeGST, the migrations of these enzymes were compared with that of a confirmed dimeric protein, human GST P1-1 (∼46 500 Da), and to a monomeric protein, trypsin inhibitor from soya bean

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(∼20 000 Da) (Fluka). All four proteins have similar pI values ranging from 4.5 to 5.5.

Sequence alignments

For similarity analyses, sequences representing GSTs from other classes were aligned with TeGST and SeGST using the BioEdit Sequence Alignment Editor software utilizing the ClustalW algorithm [26]. The upper four sequences in Figure 3 are representatives of the new type of enzyme in different cyanobacterial species. Proteins from the following species were included in the alignment (GST class and GenBank® accession numbers in parentheses) TeGST (NP_680998), SeGST (YP_171005), *Anabaena variabilis* ATCC 29413 (YP_322519), *Nostoc punctiforme* PCC 73102 (ZP_00108656), *Proteus mirabilis* (Beta) (P15214), *Escherichia coli* (P0A9D2), *Anopheles gambiae* (Delta) (CAB03593), *Ommastrephes sloani* (Sigma) (P46088), *Arabidopsis thaliana* (Zeta) (AAG30131), *Homo sapiens* (Theta 1) (NP_000844) *Homo sapiens* (Alpha 1) (NP_665683), *Homo sapiens* (Mu 1) (NP_000552), *Homo sapiens* (Pi 1) (P09211), *Homo sapiens* (Omega 1) (AAV68046) and *Homo sapiens* (Kappa 1) (EAL23783).

RESULTS

Cloning, expression and purification

Amplification by PCR yielded a single product that was evident as a sharp band for each GST, as judged by agarose gel electrophoresis (results not shown). Cloning of the PCR product was carried out in the vector pGEM3-Z before subcloning into the expression vector pKK-D. Sequencing of the cDNA showed no deviations in any of the two sequences as compared with the data in GenBank®. SDS/PAGE analysis of crude lysates from small-scale expression experiments showed marked overexpression of both proteins, but to a significantly higher level for TeGST. Both proteins bound adequately to the Glutathione Sepharose™ 4 Fast Flow matrix, but SDS/PAGE analyses (results not shown) of the flowthrough showed that a large amount of protein remained in solution after binding, even after increasing the amount of affinity gel. Pure TeGST and SeGST were both efficiently eluted by GSH in 18 and 13 ml pools respectively. Elution using glycine buffer at pH 10 was also tested successfully, but proved to affect enzymatic performance in a negative way. These batches were used for amino acid analysis. The purification yield was in the order of 150 mg of TeGST and 18 mg of SeGST from 3 litres of bacterial culture. SDS/PAGE gels stained by Coomassie Brilliant Blue also showed impeccable purity of both proteins. The analysis showed that the amino acid contents of the purified proteins were correct when compared with the sequences with GenBank® accession numbers NP_680998 for TeGST and YP_171005 for SeGST. The results obtained were also used to calculate specific absorption coefficients at 280 nm. The values were 1.6 and 1.5 $l \cdot g^{-1} \cdot cm^{-1}$ for TeGST and SeGST respectively. Proteins were kept on ice after purification, and the catalytic activities were retained for at least 4 months.

Enzyme kinetics

The specific activities are presented in Table 2. The ITC substrates were by far the best substrates tested for these enzymes, reaching over 100 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ for SeGST. Activities could be determined for several other substrates such as CDNB, CuOOH and NBD-Cl, representing different types of chemical reactions. Generally, SeGST catalysed reactions more readily than did TeGST. Catalytic constants are shown in Table 3. K_m values for GSH are within the normal range, below 1 mM, as observed for many mammalian GSTs. The K_m values for phenethyl-ITC are lower, in the micromolar range, as also observed for human GSTs. Cibacron Blue proved to be an efficient inhibitor with IC_{50} values of 0.05 μM for SeGST and 0.0075 μM for TeGST respectively. IC_{50} values for hexyl glutathione were considerably higher, 0.15 mM for SeGST and 0.5 mM for TeGST.

Table 2 Specific activities

Results are means ± S.D. of five independent measurements as described in the Experimental section. ND, not detectable, i.e. no enzymatic activity could be detected under the assay conditions used.

Substrate	Specific activity of TeGST ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	Specific activity of SeGST ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)
CDNB	0.14 ± 0.005	0.17 ± 0.01
DCNB	ND	ND
NBC	0.15 ± 0.007	0.27 ± 0.032
Ethacrynic acid	0.04 ± 0.003	0.10 ± 0.004
EPNP	0.34 ± 0.020	0.42 ± 0.020
CuOOH	0.24 ± 0.006	0.75 ± 0.013
H_2O_2	0.038 ± 0.001	ND
NBD-Cl	1.54 ± 0.062	5.26 ± 0.26
Trans-2-nonenal	0.04 ± 0.006	ND
Phenethyl-ITC	33 ± 2	102 ± 2
Allyl-ITC	14 ± 1	15 ± 0.8
Propyl-ITC	8 ± 1	6 ± 0.4
Cyclohexyl-ITC	11 ± 0.6	21 ± 0.8
Sulforaphane	5 ± 0.4	26 ± 1
Iodomethane	ND	ND
1-Iodopenthane	ND	ND
Dichloromethane	ND	ND

Table 3 Steady-state kinetic constants

Enzymatic activities were measured at various concentrations of GSH and phenethyl-ITC as described in the Experimental section. Kinetic constant calculations are based on three independent measurements for each concentration and are means ± S.D.

Substrate	Kinetic parameter	TeGST	SeGST
GSH	k_{cat} (s^{-1})	21.4 ± 0.58	63.4 ± 2.4
	K_m (mM)	0.16 ± 0.019	0.75 ± 0.090
	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	135 ± 16.2	84.6 ± 10.2
Phenethyl-ITC	k_{cat} (s^{-1})	17.0 ± 1.1	48.7 ± 2.4
	K_m (mM)	0.047 ± 0.011	0.084 ± 0.014
	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	360 ± 84	580 ± 97

Thermal stability

Specific activities towards phenethyl-ITC measured after incubation at different temperatures are presented in Figure 1. The results with CDNB were very similar (results not shown). Both proteins exhibited high thermal stability, but TeGST was more heat-resistant, with almost 100 % of its activity retained after incubation at 70 °C.

Quaternary structure determination

Figure 2 shows the non-denaturing PAGE gel. A comparison of the migration of TeGST and SeGST with the migration of human GST P1-1 and soya bean trypsin inhibitor indicates a dimeric structure of both cyanobacterial proteins. Facilities of the ExPASy Proteomics Server were used to calculate the pI values of 5.36 and 5.45 for TeGST and SeGST respectively, and these pI values were

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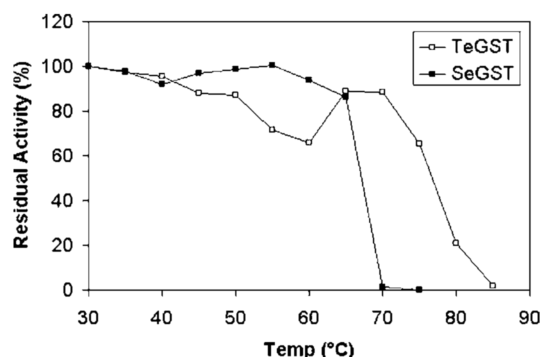

Figure 1 Thermal stability

Residual activity after incubation at different temperatures for 10 min. Activity measured after incubating samples at 30 °C was taken as 100 %. Phenethyl-ITC was used as electrophilic substrate.

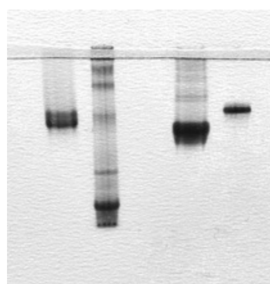

Figure 2 Quaternary structure determination by non-denaturing PAGE

Electrophoretic mobility of proteins of different masses was compared for determination of quaternary structure. From left to right, proteins are hGSTP1-1 (~46 500 Da), trypsin inhibitor from soya bean (~20 000 Da), purified TeGST and SeGST (both ~20 500 Da per monomer).

similar to the values of the reference proteins used in the electrophoresis. Monomeric trypsin inhibitor has a molecular mass of ~20 000 Da, very close to that of a single subunit of cyanobacterial GST, and dimeric human GST P1-1 has a mass of 46 500 Da. The electrophoresis clearly shows that the migration of these cyanobacterial GSTs is similar to that of GST P1-1, leading to the conclusion that TeGST and SeGST are probably dimeric proteins.

Sequence alignments

TeGST was compared with a selection of other GSTs (Figure 3). The highest sequence identities, 55 %, were displayed by SeGST as well as by other cyanobacterial GSTs. If conservative amino acid replacements are counted, the similarity is up to approx. 70–78 %. The alignment supports the grouping of these enzymes into a new class, since sequence identity with any other enzyme does not reach the 40–50 % suggested for class affiliation. The Chi class cyanobacterial GSTs show the highest identity with the Delta and Zeta class enzymes, 35 % and 32 % respectively. Identity with human GSTs is 27 % for both Theta and Pi, which is approximately the same as with the non-cyanobacterial GSTs from prokaryotes included in Figure 3. Secondary structure predictions were also performed for all GSTs in the alignment using the JPred online secondary structure prediction service (http://www.compbio.dundee.ac.uk/Software/JPred/jpred.html) [27]. The result is shown in Figure 4. A few conserved amino acids and local structural patterns have been identified in almost all GSTs identified so far. A proline residue in the N-terminal part, position 53 in cyanobacterial GSTs, is conserved in cytosolic enzymes. Also, an isoleucine residue in position 68, often preceded by an alanine residue, is, like Pro-53, invariably present as part of what has been denoted GST motif I [28]. GST motif II [28] in the C-terminal part is also present in the cyanobacterial GSTs.

DISCUSSION

GST enzymes and their variety of functions have for long attracted considerable interest. Although the family can be traced back to the beginning of life, GSTs from prokaryotes, with a few exceptions, have not emerged as research targets until recently. In the present study, cyanobacterial GSTs are studied for the first time, and this work has led to the introduction of a new class of cytosolic GSTs, the Chi class. In contrast with previous studies on bacterial GSTs, the results presented here show that a GST from an evolutionarily less diverged cell can have functions of both variety and specificity.

The alignment in Figure 3 shows that typical structural features found in all known cytosolic GSTs are also present in the cyanobacterial enzymes. One example is the strictly conserved Pro-53 that adopts the *cis*-configuration, as judged by crystal data obtained for other GSTs. Mutagenesis of this residue gave different effects depending on the GST studied, as exemplified by PmGST B1-1 [29] and human GST A1-1 [30]. Other examples are the two segments of amino acids defined as GST motif I (residues 49–73 in TeGST and SeGST) and GST motif II (residues 130–147 in TeGST and 129–146 in SeGST respectively) identified in GSTs as well as in some non-GST proteins [28]. The importance of, in particular, GST motif II for structure and folding has been convincingly demonstrated in several studies. Within this motif, the local structural motifs, denoted N-capping box and hydrophobic staple, are crucial for the folding of GSTs, as has been shown for human GSTs [31–33], and lately also for bacterial GST from *Burkholderia* sp. LB400 [34] and PmGST B1-1 [35]. For prokaryotic GSTs, these residues may also contribute to catalysis, as exemplified by mutational analysis of the GST from *Burkholderia* [34]. Otherwise, no particular part of the cyanobacterial sequences can be found in other GSTs. There are bacterial genes annotated as GSTs in GenBank® that are approx. 35 % identical with the cyanobacterial GSTs, but, since nothing is known about these putative enzymes, they were not included in the alignment in Figure 3. A catalytically important tyrosine, serine or cysteine residue is usually located in the N-terminus of GSTs. There are several possible candidates in the cyanobacterial GSTs studied here, but final identification of catalytic residues has to await crystal structure determination and structure–function analysis.

GSTs are considered to have evolved from thioredoxin, a small monomeric protein [36]. The thioredoxin origin is maintained as a domain in the tertiary structure of GSTs, as shown in numerous crystal structures. Typical for thioredoxins is the presence of two catalytic cysteine residues [36], which are also conserved in bacterial Beta class GSTs. The function of these cysteine residues has been studied in the prototypic bacterial enzyme PmGST B1-1, and the results pointed to a role of the cysteine residues in the original function as a redox enzyme [12]. Surprisingly, the two cyanobacterial GSTs described here lack cysteine residues completely, as do other cyanobacterial GSTs confined to the new Chi class. This may be an indication of a different evolutionary pathway for the cyanobacterial GSTs from the *Proteus mirabilis* enzyme. The lack of the two cysteine residues and the low sequence identity between the cyanobacterial enzymes presented here and other bacterial representatives excludes the incorporation of the former into the Beta class GSTs. Secondary structure

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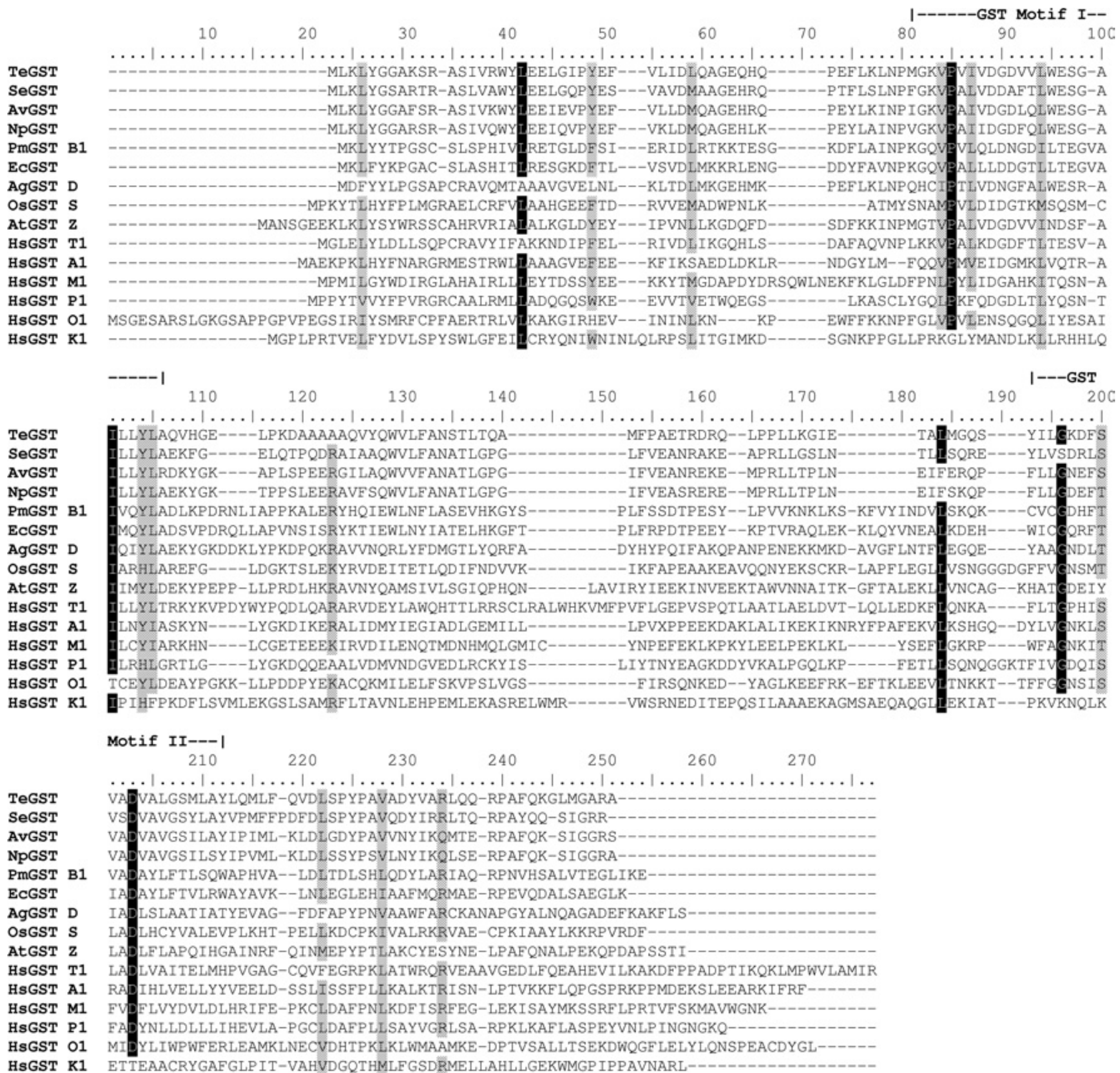

Figure 3 Alignment of complete amino acid sequences representing GSTs from various sources

TeGST and SeGST aligned with representatives from human, plant, proteobacterial and other cyanobacterial GSTs. A black background indicates identities, and similar amino acids are shaded grey. The locations of GST structural motifs I and II are indicated above the sequences. Sequence aliases with corresponding accession numbers are TeGST (NP_680998), SeGST (YP_171005), AvGST [*Anabaena variabilis* ATCC 29413 (YP_322519)], NpGST [*Nostoc punctiforme* PCC 73102 (ZP_00108656)], PmGST B1 [*Proteus mirabilis* (P15214)], EcGST [*Escherichia coli* (P0A9D2)], AgGST D [*Anopheles gambiae* (CAB03593)], OsGST S [*Ommastrephes sloani* (P46088)], AtGST Z [*Arabidopsis thaliana* (AAG30131)], HsGST T1 [*Homo sapiens* (NP_000844)], HsGST A1 [*Homo sapiens* (NP_665683)], HsGST M1 [*Homo sapiens* (NP_000552)], HsGST P1 [*Homo sapiens* (P09211)], HsGST O1 [*Homo sapiens* (AAV68046)] and HsGST K1 [*Homo sapiens* (EAL23783)].

prediction analysis was also performed for comparison with GSTs of known three-dimensional structures (Figure 4). The results show the same secondary structure pattern as found in the cytosolic GSTs, indicating that cyanobacterial GSTs belong to this GST family. Typical characteristics are the organization into two distinct domains where the N-terminal domain I adopts the thioredoxin $\beta\alpha\beta\alpha\beta\beta\alpha$ fold, whereas the C-terminal domain is all helical. The result was the same for all cyanobacterial sequences of similar lengths annotated as GSTs, underlining the relationship between these enzymes and supporting the introduction of a new class.

The difference in expression level between TeGST and SeGST probably reflects the lower stability of SeGST. The lower thermal stability of SeGST is demonstrated by the heat inactivation experiments, although both proteins exhibit high thermal resistance. This is not unique for the cyanobacterial GSTs: high structural stability has been observed for many bacterial proteins. Recently, a study on PmGST B1-1 was presented in which structural stability was measured by means of thermal inactivation [35]. It proved to be very stable, with 80 % of its activity retained after 15 min at 70 °C. The thermal stability of TeGST is shown to be even higher, with the activity declining only slowly with increasing temperature. The difference in thermal stability between the cyanobacterial enzymes is not surprising, since they derive from organisms that have different optimal growing temperatures. But, considering the sequence and functional similarities between the cyanobacterial proteins, the difference is somewhat unexpected. Characteristics typically attributed to heat-stable proteins, such as a high content of hydrophobic and charged amino acids, do not differ significantly between the two proteins. However, the amino acid residues that may contribute to heat stability are positioned differently, which may explain the stability difference.

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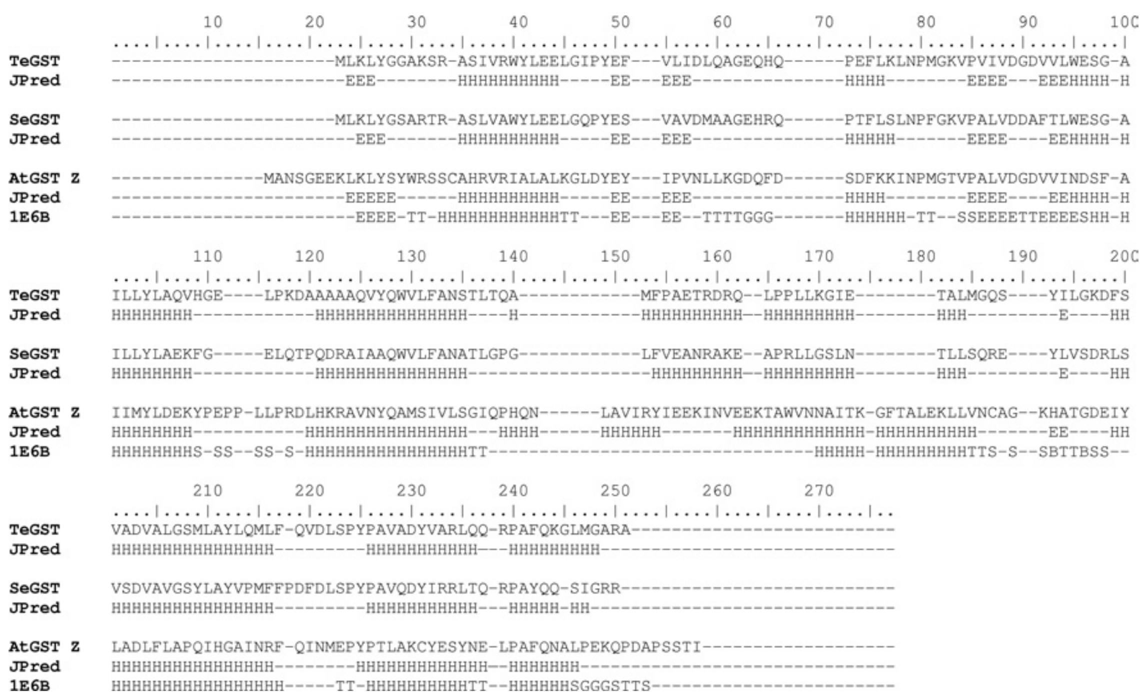

Figure 4 Secondary structure predictions with comparative crystal data for Zeta class GST

Amino acid sequences for TeGST, SeGST and AtGST are extracted from Figure 3 for presentation of secondary structure predictions. For comparison and to confirm the accuracy of JPred, structural data for Zeta class enzyme with PDB code 1E6B (*Arabidopsis thaliana*) is added below the JPred results. Structural elements are indicated using the following letters: B; β-bridge; E, extended strand; G, 3_{10} helix; H, α-helix; S, bend; T, turn. A dash indicates that structural data are not available or that the alignment algorithm has inserted a gap.

Both GSTs exhibited high storage stability of the pure proteins as both enzymes retained full activity after 4 months at 0 °C. As has been observed for other proteins, the higher thermal stability of TeGST as compared with SeGST comes at the expense of a lower catalytic efficiency.

Distinct catalytic differences between the two enzymes were also found. This is most pronounced by the results of the inhibition experiments, which can indicate somewhat different active sites in the two enzymes. Cibacron Blue is generally a strong inhibitor of GSTs, and here the effect is more pronounced for TeGST, displaying a 7-fold lower IC_{50} value than for SeGST. With the less potent inhibitor hexyl glutathione, the situation was reversed, with an IC_{50} value 3-fold lower for SeGST than for TeGST. In the context of ligand affinities, it is worth mentioning the ease with which these enzymes could be purified using a GSH-affinity matrix, which has been troublesome with many bacterial GSTs.

Substrate profiles are very similar for the two enzymes. SeGST is generally the more efficient enzyme with the tested substrates. Specific activities are modest for many of the substrates, but in the same range as has been observed for other bacterial GSTs. Typical is also the reasonably high activity with NBD-Cl. It is of the same order of magnitude as for PmGST B1-1, for which this is the best substrate identified [12]. Truly remarkable is the activity with ITCs, which is on such a high level that physiological importance seems evident. This is emphasized by the fact that the cyanobacterial GSTs can conjugate structurally different ITCs. Both linear and cyclic aliphatic as well as aromatic groups in the ITCs are accepted. GSTs from less evolved species are thus also promiscuous, a typical property of the GST enzymes. Catalytic activity towards functional groups, in contrast with specificity towards a particular substrate molecule and its structure, is also characteristic for detoxification enzymes. Various ITCs have been previously shown to be good substrates for human GSTs of different classes. Meyer et al. [14] analysed GSTs for activity with aromatic ITCs and Kolm et al. [23] tested 14 different ITCs with four human GSTs. GST M1-1 and GST P1-1 were the most efficient enzymes, and the highest specificity constants were obtained with aromatic substrates. In many later studies, ITCs have often been included as a standard substrate. In the present study, kinetic constants for phenethyl-ITC were determined in the presence of 1 mM GSH as described previously [23]. For the specific activity measurements, GSH was increased to 4 mM to obtain saturation. If k_{cat}/K_m values are calculated from the specific activity values, the values are $479\ s^{-1} \cdot mM^{-1}$ and $831\ s^{-1} \cdot mM^{-1}$ for phenethyl-ITC for TeGST and SeGST respectively. Cyanobacterial GST activities are thus at the same levels as those of the most efficient catalysts among the human GSTs with these substrates [23,37].

The fundamental question is why cyanobacteria would evolve enzymes capable of conjugating ITCs with such high efficiency. ITCs are a diverse group of chemical compounds, of which more than 100 are known, that are mainly found in plants [38]. Glucosinolates synthesized from amino acids store ITCs in an inactive form. The action of specific plant enzymes, myrosinases, cleaves glucosinolates and release ITCs together with other breakdown products. ITCs are considered to be one part of the chemical defence that plants make use of in response to injury or stress caused by pathogens or to suppress growth of competing plants. ITCs have antimicrobial activities, but the effect is highly species-dependent, and many pathogens are obviously resistant towards these compounds. The mechanism behind the resistance is largely unknown [39–41]. Recently, it was shown that the expression of a GST was induced in *Alternaria brassicicola*, a pathogenic fungus, by addition of benzyl-ITC to the growth medium [42]. It was also shown that the induced GST could catalyse the conjugation between GSH and ITCs, although at

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a significant lower rate (1–2 %) than the cyanobacterial GSTs described in the present study. GSTs may have a role in plant resistance towards endogenous ITCs. Modest catalytic activities up to 2 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} have been recorded with plant GSTs from wheat and soya bean [43,44]. Considering the proposed model for plant cell evolution, the endosymbiotic theory, it can be noted that cyanobacterial GSTs display somewhat higher sequence identities with a Zeta class plant enzyme than with most other cytosolic GSTs. However, the difference is small, and identities with other Zeta class enzymes are at the same level as with other soluble GSTs. Further studies on the many functionally diverse plant GSTs [45] could reveal whether the catalytic activities observed here are, in fact, spread among plants and evolutionarily conserved.

Cyanobacteria encompass a large and heterogeneous group of phototrophic bacteria. They are widespread in Nature in both terrestrial and marine habitats and some are extremophiles. ITCs have been identified in a few cyanobacterial strains [46] and in some marine sponges [47], but these are structurally completely different from those in the present study. Myrosinases are not exclusively plant enzymes, but have also been demonstrated in some intestinal bacterial strains. Whether myrosinases are more generally distributed and are to be found in other micro-organisms remains to be investigated. Considering the catalytic efficiency displayed by TeGST and SeGST in the conjugation reaction between GSH and ITC and the presence of corresponding similar GSTs in other cyanobacterial strains, it does seem reasonable to assume that cyanobacteria are, or have been, naturally exposed to ITCs.

In an excellent review 10 years ago, the author posed the somewhat provoking question: "Bacterial glutathione S-transferases: what are they good for?" [7]. The rationale behind the formulation was the fact that no particular or unique function or catalytic activity could be attributed to bacterial GSTs. They were abundant proteins, but much less efficient enzymes than other previously known GSTs. In the present paper, for the first time, data are presented that show that bacterial GSTs are indeed good at something and that this something probably is of true physiological importance. But, the question still remains, "What is this catalytic activity good for?"

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