A Ligand Function of Glutathione *S*-Transferase

Barbara Lederer and Peter Böger*

Department of Plant Physiology and Biochemistry, University of Konstanz, D-78457 Konstanz, Germany. E-mail: peter.boeger@uni-konstanz.de

* Author for correspondence and reprint requests

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Glutathione *S*-transferases (GSTs) are ubiquitous enzymes and abundant in plants. They are intimately involved in plant metabolism and stress defense related to reactive oxygen species. Our project assigned particular reactions including novel ones to certain GST-isoforms. Transformed *E. coli* was used to express recombinant GST-isoforms from maize. An *N*-terminal His tag allowed their purification by affinity chromatography. Three GST-monomers had a molecular weight of 26, 27, 29 kDa, and aggregated to dimers when assayed for their enzymic properties. Four dimeric isoforms were used to study how they interact with tetrapyrroles (of the chlorophyll biosynthesis pathway). It was found that protoporphyrin IX (Proto IX), Mg-protoporphyrin and other tetrapyrroles are bound non-covalently ("liganded") to GSTs but not conjugated with reduced glutathione. This binding is non-covalent, and results in inhibition of conjugation activity, the degree depends on type of the porphyrin and GST-isoform. I_{50}-values between 1–10 μM were measured for Proto IX, the inhibition by mesoporphyrin and Mg-protoporphyrin was 2- to 5-fold less. The ligand binding is non-competitive for the substrate 1-chloro-2,4-dinitrobenzene and competitive for glutathione. The dimer GST 26/26 prevents the (non-enzymic) autoxidation of protoporphyrinogen to Proto IX, which produces phytotoxic reactive oxygen species in the light. GST 27/27 protects hemin against degradation. Protoporphyrinogen is formed in the plastid and then exported into the cytosol. Apparently binding by a suitable GST-isoform ensures that the highly autoxidizable protoporphyrinogen can safely reach the mitochondrium where it is processed to cytochrome.

Key words: Ligand Function, Glutathione *S*-Transferase, Protection of Protoporphyrinogen

Introduction

Glutathione *S*-transferases (GSTs, EC 2.1.5.18) are ubiquitous enzymes, conjugating hydrophobic, electrophilic compounds with glutathione (GSH) by covalent binding. In plants, particularly toxic substrates are thereby tagged for vacuolar import assisted by ATP-binding transporters (Gaillard *et al.*, 1994; Edwards *et al.*, 2000). GSTs play a major role in stress defense related to reactive oxygen species. They often make up 1% of the total soluble plant protein. We studied the characteristics of four dimeric isoforms from corn and found them differentially active for glutathione (GSH)-mediated conjugation of herbicides (like metazachlor, a chloroacetamide), for enzymic degradation of toxic unsaturated aldehydes or for peroxidase activity. Even a catalytic isomerization has been reported. Some activities of GSTs are listed in Table I while Fig. 1 demonstrates by some examples particular reactions ascribed to certain GST-isoforms.

Only a few naturally occurring substrates of GSTs have been unequivocally identified. Phenylpropanoids like cinnamic or coumaric acid (Dean *et al.*, 1995), or auxins (Watahiki *et al.*, 1995) are bound to specific GSTs without GSH-conjugation (Mueller *et al.*, 2000). It is assumed that besides their GSH-conjugation activity a major role of GSTs in plants is their property to act as binding proteins (Alfenito *et al.*, 1998; Walbot *et al.*, 2000). In animal cells, it has been shown that GSTs bind heme, protoporphyrin IX (Proto IX), and biliverdin (Smith, 1987). Certain GSTs were termed "ligandins" rather than "transferases" due to their ability to non-covalently bind bilirubin and other toxic metabolites with high affinity (Litwack *et al.*, 1971; Habig *et al.,* 1974). Apparently plant GSTs can function as carriers for physiologically relevant porphyrins, but fundamental biochemical data from plants concerning this assumption are missing.

Porphyrin biosynthesis in plants requires a cross-talk between the organelles. One central tetra-

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Table I. What do glutathione *S*-transferases perform?

Process	Conjugation with GSH
1) Dechlorination of xenobiotics (atrazine, chloroacetamides)	yes
2) Split xenobiotics with the same mode of action as chloroacetamides (fentrazamide, cafenstrole) – assumed	yes
3) Break up the oxirane ring of xenobiotics (tridiphane, indanofan)	yes
4) Split the ether bridge of (some) diphenyl ethers (acifluorfen, fluorodifen)	yes
5) Convert toxic unsaturated carbonyls (*e.g.* crotonaldehyde)	yes
6) Isomerization GSH (thiadiazolidines to triazolidines)	GSH-intermediate
7) Deactivate peroxides (*e.g.* linolenic acid hydroperoxide)	no
8) Hydrolysis of esters (fenoxaprop-ethyl) – assumed side activity	no
9) Cytoplasmic carrier protein for flavonoids, anthocyanins	no
10) Association with porphyrins: Protection of protoporphyrinogen against oxidation	no

to (1): Hathway (1989). Marrs (1996)
to (3): Lamoureux and Rusness (1986).
to (4): Frear *et al.* (1983).
to (5): Cummins *et al.* (1999); Sommer and Böger (1999, 2001).
to (6): Nicolaus *et al.* (1996).
to (7): Cummins *et al.* (1999); Sommer and Böger (1999, 2001).
to (8): J. C. Hall, pers. commun.
to (9): Mueller *et al.* (2000).
to (10): this contribution.

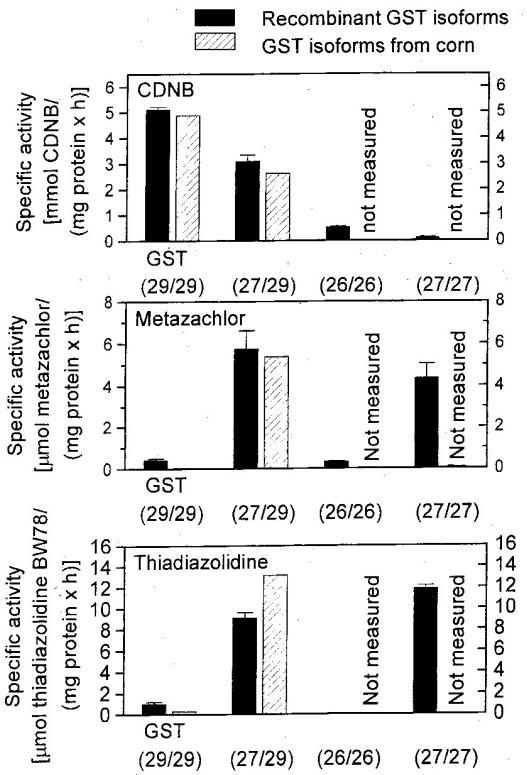

Fig. 1. Specific activities of four dimeric isoforms from corn using three different substrates. Upper and middle part: conjugation of CDNB or metazachlor, respectively (Sommer and Böger, 1999). Bottom part: Isomerization of a thiadiazolidine to a triazolidine (Nicolaus *et al.*, 1996). Note that activities of the recombinant enzymes (black columns) are in accordance with the natural ones (hashed columns). Metazachlor is 2-chloro-*N*-(2,6-dimethylphenyl)-*N*-(1*H*-pyrazol-1-ylmethyl)acetamide.

pyrrole precursor, protoporphyrinogen (Protogen), is synthesized exclusively in the chloroplast (Beale, 1999; Fig. 2). Compartmentation of the tetrapyrrole pathway requires interorganelle transport and protective mechanisms in the cytosol. Plastid-derived Protogen is hydrophilic and nontoxic but in the cytosol it is readily oxidized to the lipophilic and phytotoxic Proto IX. Its photoactivation produces reactive oxygen species resulting in peroxidative damage. This detrimental property determines the mode of action of peroxidizing herbicides (Wakabayashi and Böger, 1999), imposing the necessity for a regulation of Proto IX formation.

In the chloroplast and the mitochondrion, Proto IX is converted to the ferrous protoporphyrin heme. Heme serves as a cofactor of numerous cytosolic enzymes, therefore its transfer to the cytosol and/or appropriate organelle is assumed to produce the mature enzymes (Werck-Reichardt *et al.*, 1988). A steady efflux of heme from chloroplasts into the cytosol does occur (Thomas and Weinstein, 1990), but little is known about the appropriate transport and assembly to apoproteins. In animals, some evidence indicates GSTs to act as heme-transfer proteins (Senjo *et al.*, 1985). The importance of plastidic and cytosolic tetrapyrrole allocation in plants has been demonstrated by a mutant with deregulated porphyrin synthesis due to lack of a protoporphyrin(ogen)-dependent ABC-transporter (Mϕller *et al.*, 2001). Nevertheless, knowledge on intracellular porphyrin transport in plants is rare. This study written as a minireview will provide further insights for the activity of plant GSTs.

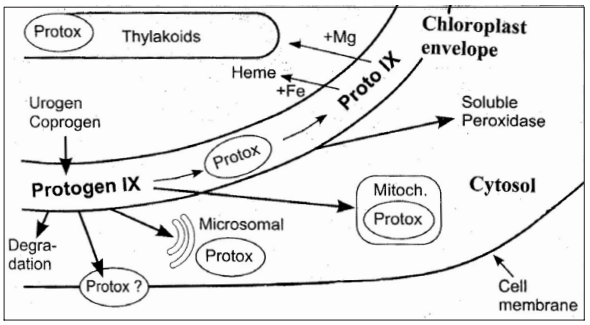

Fig. 2. Protoporphyrinogen (= Protogen) is produced in the chloroplast and then exported into the cytosol. Its oxidation may occur by autoxidation or enzymatically. "Protox" (encircled) indicates the sites for enzymic oxidation by protoporphyrinogen oxidase, forming protoporphyrin IX (= Proto IX). Urogen, coprogen denote uroporphyrinogen and coproporphyrinogen, respectively, are precursors of Protogen.

Materials and Methods

Experimental details have been published elsewhere. For preparation of plasmids to genetically transform *E. coli* see Sommer and Böger (1999, 2001). More details on the enzymology and assays of GST-isoforms can also be found in these references. Fluorescence measurements on porphyrins are described by Lederer and Böger (2003). The GST-dimers are denoted by the molecular weight of the particular monomers (in kDa) to facilitate comparison with our previous publications.

Results and Conclusion

Porphyrins bind to GSTs

The GST-isoforms did not degrade Proto IX after binding. Intensive analysis of spectroscopic shifts in Proto IX absorbance, of fluorescence emission or excitation spectra, or our chromatographic separation studies of Proto IX-GSH derivatives by HPLC did not provide any evidence for a GSH-conjugation with Proto IX. Purified GSTs were preincubated briefly (5 min) with different porphyrins and were subjected to gel electrophoresis under non-denaturating conditions conserving their native structure during separation. GSTs bind Proto IX as was demonstrated by the clear migration of bound porphyrin together with each of the examined isoforms. Non-bound Proto IX alone moved only a very small distance to the anode. Heated GST-isoforms or other proteins did not bind Proto IX. All GST-isoforms were able to bind mesoporphyrin, coproporphyrin, uroporphyrin and Mg-protoporphyrin (Mg-Proto IX).

Inhibition of enzymatic activity by porphyrins

All GST-isoforms performing GSH conjugation with CDNB (1-chloro-2,4-dinitrobenzene) were inhibited. Each isoform showed a linear reduction of activity with increasing Proto IX concentration, 50 μM resulted in total inhibition (comp. Lederer and Böger, 2003). As a control, catalase was not inhibited by Proto IX (data not shown). The apparent I_{50}-value was found between 1 to 5 μM for GST 29/29, for GST 26/26 between 5 to 10 μM, and between 10 to 25 μM for GST 27/27 (see Sommer and Böger, 1999 for general enzymatic properties of the corn GST-isoforms). Proto IX at a concentration of 25 μM inhibited the activity by 91% to 99%, while mesoporphyrin (25 μM) or Mg-Proto IX (25 μM) inhibited the isoforms to a smaller extent than the metal-free Proto IX. The diphenyl ether oxyfluorfen, whose molecular size is about half of a porphyrin molecule (Nandihalli *et al.,* 1992), inhibited GSTs as well (I_{50}-value about 50 μM).

Measured with GSH and CDNB as substrates, the inhibition of GST 27/29 by Proto IX was found to be noncompetitive against CDNB (Fig. 3, left side). Apparently, the binding site of CDNB is not occupied by the porphyrin. In contrast, data plots with different GSH levels (Fig. 3, right side) indicated a competitive inhibition of GST 27/29 by Proto IX with respect to GSH. GST 29/29 and GST 27/27 exhibited the same type of inhibition (data not shown).

Protection of protoporphyrinogen against oxidation

A rapid nonenzymatic oxidation of the nonfluorescent Protogen to the fluorescent Proto IX takes place under physiological conditions *in vitro.* In air-saturated buffer at pH 7, Protogen was converted to Proto IX (Fig. 4). Addition of GST 27/27 or bovine serum albumin (BSA) did not alter the autoxidation significantly, but the GST 26/26 dimer substantially prevented Protogen autoxidation. Even low GST concentrations could delay the exponential acceleration of Proto IX formation. Autoxidation of the surplus Protogen in the reaction mixture occurred after longer incubation (data not shown). The protective effect of the GST 26/26 homodimer on Protogen autoxidation indicates a functional specificity, since neither BSA

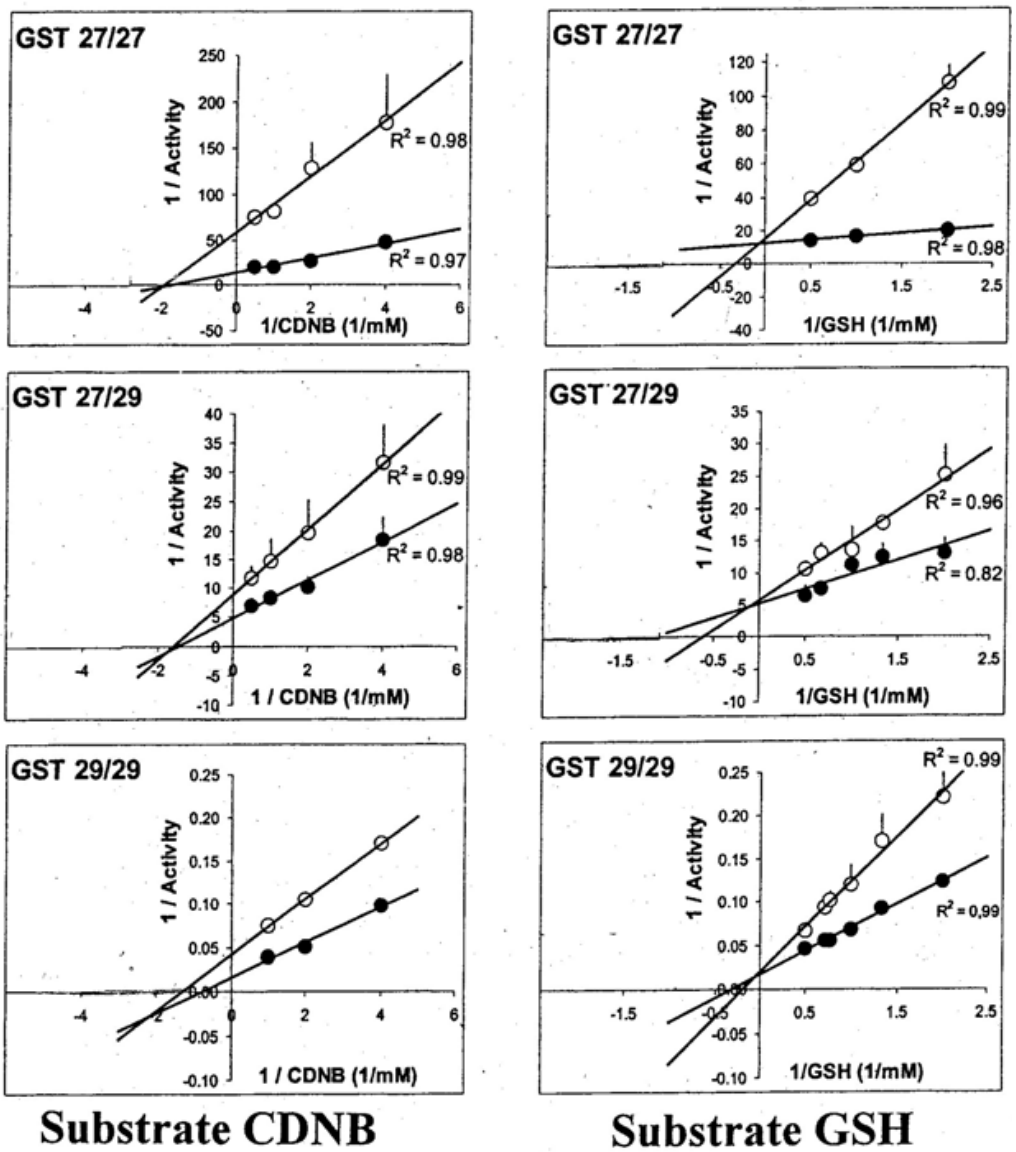

Fig. 3. Double-reciprocal plots of activities of three dimeric GST-isoforms inhibited by Proto IX (for nomenclature the kDa molecular weights of the monomers are used, see Fig. 1); left side with varying CDNB concentrations; right side with varying GSH. For experimental details see Lederer and Böger (2003). ●: no Proto IX; ○: with 5 μM Proto IX.

nor the other GSTs of this study tested could protect Protogen to this extent.

Hemin degradation impaired

Only a small decrease of hemin absorbance was observed with hydrogen peroxide or GSH present alone. However, adding both GSH and hydrogen peroxide hemin was rapidly degraded. GST-isoforms were tested for their ability to prevent this degradation. Addition of the GST 27/27 homodimer to heme solutions caused a spectral shift to longer wavelengths in the maximum band of hemin, indicating interaction with the tetrapyrrole (Fig. 5B and C). This shift was not observed with the other GST-isoforms or BSA (data not shown).

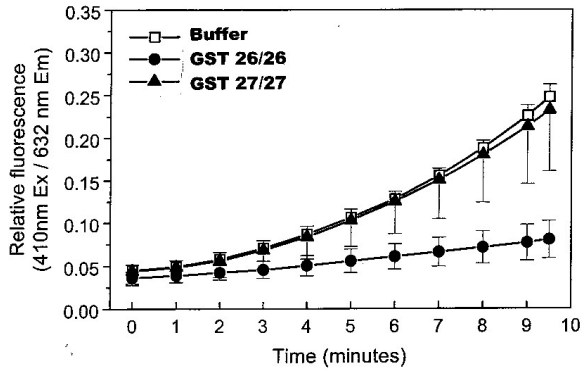

Fig. 4. Autoxidation of protoporphyrinogen IX to protoporphyrin IX in oxygen-saturated medium measured by fluorescence of Proto IX. Oxidation was prevented by GST 26/26 (lower curve) while GST 27/27 showed no effect (upper curve, identical with the control).

GST 27/27 protected hemin against oxidative degradation (Fig. 5A). The decrease of hemin with GST 27/27 present was only 54% compared to the decrease of the control in buffer set to 100%. Also the other GST-isoforms exhibited some protection although less than GST 27/27.

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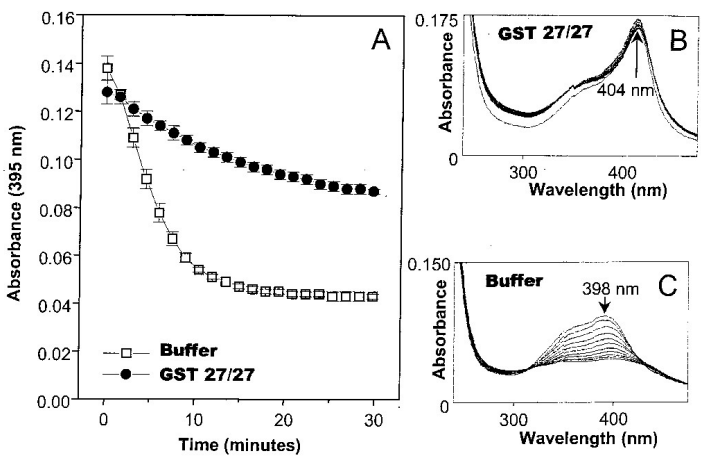

Fig. 5. Degradation of hemin assayed with 0.2 μM hemin at pH 7.0; the reaction mixture included 1 mM GSH and 10 μM H_2O_2 with 1 μM GST, or the appropriate volume of protein storage buffer. (A) Decrease of hemin absorbance at 395 or 404 nm, (B) time-dependent change of hemin spectra with GST 27/27, or (C) with bovine serum albumin added.

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