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Interaction of Glutathione Transferase P1-1 with Captan and Captafol

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ABSTRACT. Glutathione transferase (GST, EC 2.5.1.18) P1-1 was strongly inhibited by captan and captafol in a time- and concentration-dependent manner. The IC_{50} values for captan and captafol were 5.8 μM and 1.5 μM, respectively. Time-course inactivation of GSTP1-1 by two pesticides was prevented by 3 mM of hexylglutathione, but not by methylglutathione. The fact that the inactivated enzyme recovered all the 5,5′-dithiobis(2-nitrobenzoic acid) titrable thiol groups, with concomitant recovery of all its original activity after treatment with 100 mM dithiothreitol, suggested that captan and captafol were able to induce the formation of disulfide bonds. That the inactivation of GSTP1-1 by captan and captafol involves the formation of disulfide bonds between the four cysteinil groups of the enzymes was confirmed by the SDS-PAGE experiments on nondenaturant conditions. In fact, on SDS-PAGE, GSTP1-1 as well as the cys47ala, cys101ala, and cys47ala/cys101ala GSTP1-1 mutants treated with captan and captafol showed several extra bands, with apparent molecular masses higher and lower than the molecular mass of native GSTP1-1 (23.5 kDa), indicating that both intra- and inter-subunit disulfide bonds were formed. These extra bands returned to the native 23.5 kDa band with concomitant restoration of activity when treated with dithiothreitol. BIOCHEM PHARMACOL 52;1:43–48, 1996.

KEY WORDS. GSH; GST; captan; captafol; pesticides; interaction

Cytosolic GSTs§ (EC 2.5.1.18) comprise a family of multifunctional dimeric proteins that catalyze conjugation of glutathione to a large variety of endogenous and exogenous electrophilic compounds [1, 2]. GST are also involved in intracellular binding and transport of hydrophobic molecules, such as heme, bile acids, bilirubin, and polycyclic hydrocarbons, participate in prostaglandin and leukotriene biosynthesis, and seem to play a key role in the elimination of toxic hydroperoxides [1–3]. Moreover, GST may be implicated in cell-line resistance to anticancer drugs, herbicides, and pesticides [1–4].

Several investigations have dealt with the structure and function of cytosolic GST, and the multitude of isoforms characterized to date have been grouped into at least 5 distinct classes, namely alpha, mu, pi, theta, and sigma [5–7]. An additional class is represented by microbial GST [8, 9]. All cytosolic GSTs are homo- or hetero-dimers of subunits whose molecular masses are in the 23–27 kDa range [1, 2]. According to X-ray crystallographic analysis, alpha, mu, pi, theta, and sigma GST monomers show an overall similar structural organization (i.e., two domains joined by a short linker of six or seven amino acid residues) [10–14]. Domain I (GSH-binding site), which is mainly located in the N-terminal region of the protein, is an α/β structure [10–14]. GST domain II provides most of the hydrophobic binding sites (H-site), and is formed by α-helices [10–14]. GSTP1-1 is a pi class enzyme, having an isoelectric point at pH 4.6, formed by two identical catalytic subunits with a molecular mass of 23 kDA. Four cysteine residues are present in each subunit. X-ray crystal studies have revealed that none of the four cysteine residues of the GSTP1-1 subunit form GSH- or substrate-binding sites [10, 13]. Furthermore, site-direct mutagenesis of cysteine residues strongly suggests that they are not involved in catalysis [15, 16]. However, several agents are known to induce intrachain disulfide bridge formation, producing a covalent modification of cysteine residues with GSTP1-1 inactivation [17, 18]. Using the 2-p-toluidinylnaphthalene-6-sulphonate (TNS) binding fluorescence inhibition technique, we have recently shown that various classes of pesticides are able to bind, with a relatively low affinity, GST belonging to alpha, mu, and pi classes [19]. On the other hand, the substituted phthalimides, captan and captafol, when added to the TNS-GST complex, produced an increase of fluorescent intensity with a concomitant blue-shift [19]. This phenomenon was probably due to an in-

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§ *Abbreviations:* GST, glutathione transferase; GSH, reduced glutathione.

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crease of the hydrophobicity of the TNS site, as a result of conformational changes induced by pesticide action [19]. Captan and captafol are important surfase protectants used to control a broad spectrum of plant pathogenic fungi that have been demonstrated to be able to oxidize, *in vitro*, low-molecular-weight thiol compounds [20, 21]. The present study was designed to provide further insight into the possible mechanism of interaction of GST P1-1 with captan and captafol (Fig. 1).

Evidence is presented indicating that captan and captafol inactivate GSTP1-1 acting as an oxidizing agent of the isoenzyme sulphydryl groups.

MATERIALS AND METHODS

Materials

Methylglutathione, hexylglutathione and 1-chloro-2,4-dinitrobenzene were obtained from Aldrich (Beerse, Belgium). Captan and captafol were purchased from Società Italiana Chimici (Rome, Italy). All other reagents were of analytical grade.

Enzyme Purification and Assay

GSTP1-1 was purified from human placenta as previously reported [22]. Construction of mutants, purification, and check of purity were done as previously described [23]. After affinity purification, the mutant enzymes were all homogeneous as judged by reverse-phase HPLC and SDS/PAGE. The retention time in the HPLC system and the apparent molecular mass (23 kDa) were identical to those of the wild-type enzyme. The specific activity of GSTP1-1 as measured with 1-chloro-2,4-dinitrobenzene was 85 U/mg. The specific activity of the mutants was 54 U/mg for the cys47ala GST mutant, 109 U/mg for the cys101ala GST mutant and 53 U/mg for the cys47ala/cys101ala GST mutant.

Enzyme activity was measured using 2 mM GSH and 1 mM 1-chloro-2,4-dinitrobenzene as substrate, in 0.1 M potassium phosphate buffer, pH 6.5, 1 mM EDTA [24]. Protein concentration was determined by the Bio-rad assay, using bovine γ-globulin as standard [25].

Inactivation of GSTP1-1 and GSTP1-1 Mutants by Pesticides

Captan and captafol were allowed to react with GSTP1-1 and GSTP1-1 mutants as follows. About 15 μM of GSTP1-1 or GSTP1-1 mutants were incubated at 24°C with captan or captafol (1–100 molar ratio) in the presence of 0.01 M potassium phosphate buffer, 1 mM EDTA pH 7.0–6.5, and 0.01 M 2-[N-morpholino]ethanesulfonic acid pH 5.8–5.0. At various times, 1-μL aliquots were assayed for GST activity as described above. No pesticides were added in the control cuvettes. Pesticides and their byproducts were, then, removed by passing the isoenzymes through a G-25 Sephadex column (Pharmacia NAP-5 column), and incubated with 100 mM dithiothreitol.

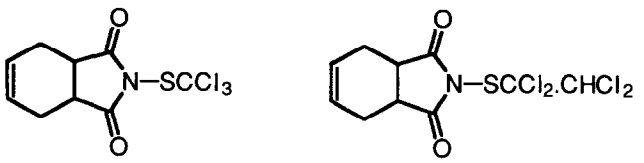

FIG. 1. Structures of pesticides.

Thiol Group Titration

Protein sulphydryl groups were measured essentially by Ellman's procedure [26]. Approximately 100 μg of GSTP1-1 was reacted with 0.1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in 1.0 mL final volume of 0.1 M potassium phosphate buffer, pH 8.0, 1 mM EDTA. The reaction was followed spectrophotometrically at a continuous 412 nm.

Electrophoresis

SDS/PAGE was performed on a Phast-system instrument (Pharmacia, Sweden) using 20% precast acrylamide minigels (PhastGel, homogeneous 20) as separating media. Conditions for separation, staining, and destaining gels were according to manufacturer's instructions.

RESULTS AND DISCUSSION

The effect of captan and captafol on GSTP1-1 activity is presented in Fig. 2. GSTP1-1 was inactivated with increasing concentrations of pesticides. 50% inactivation of GSTP1-1 activity was obtained with 1.5 μM of captafol

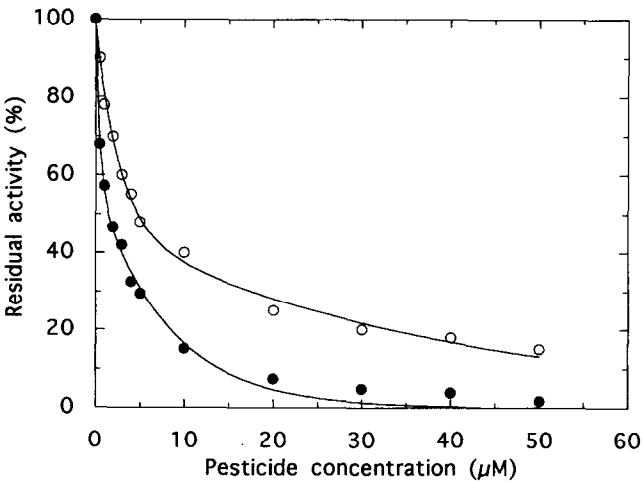

FIG. 2. Inhibition of GSTP1-1 activity by captan (○) and captafol (●). The reaction mixtures, containing 0.1 M potassium phosphate buffer pH 7.0, 1 mM EDTA, 2 mM GSH and GSTP1-1 (1 μg) in a total volume of 2 mL, were preincubated with pesticide for 30 sec at 25°C before the addition of 1 mM 1-chloro-2,4-dinitrobenzene.

and 5.8 μM of captan. This indicates that both pesticides strongly interact with GSTP1-1. Figure 3 shows the time-course of inactivation of GSTP1-1 by captan and captafol, at a 1:1 pesticide:enzyme molar ratio carried out in the 5.0–7.0 pH range. Due to the instability of substituted phthalimides at higher pH values, no inactivation experiments could be done in alkaline solution [21]. Furthermore, in this range of pH, it also prevented the "spontaneous" GSTP1-1 inactivation from occurring, in the presence of catalist, in alkaline solution [17]. Under our experimental conditions, pH-independent inactivation due to a solvation of GSTP1-1 occurring at low enzyme concentrations [27] is negligible, as appears from the results reported in fig. 3. Captafol was more inhibitory than captan, demonstrating 75–80% inhibition after 4 min of incubation at pH 7.0. Captan was less inhibitory, reaching approximately 70% of inhibition at a 1:1 pesticide:enzyme molar ratio after 60-min incubation. When the experiments were done at lower pH values, a lower inactivation rate was obtained for both captan and captafol, indicating that the time-course of GSTP1-1 inactivation is affected by the pH values. This result suggests that cys47, which has a pK value at approximately pH 4.5 [28], might be implicated, in its unprotonated form, in the inactivation of GSTP1-1. The pesticide-inactivated enzyme completely recovered its initial activity after treatment with 100 mM dithiothreitol (Fig. 3). All these data point to the probable involvement of a number of other GSTP1-1 cysteine residues in addition to cys47, in a reversible redox process, as was hypothesized for the GST7-7 and GST π, which undergo a similar reversible inactivation in the presence of H_2O_2 [18]. In this context, it is worthy of mention that both captan and captafol are able to oxidize low-molecular-weight thiol compounds *in vitro* [20, 21]. GSTP1-1 inactivation was partially prevented by 3 mM of hexylglutathione, whereas a very low protection was seen with 3 mM of methylglutathione (Fig. 4). These results suggest that mixed disulfide formation could also involve cysteine residues far from the G-site. GSTP1-1 contains four cysteine residues per monomer (i.e., cys14, cys47, cys101, and cys169) [29]. However, only three residues per monomer are titrable with specific reagents [17]. Therefore, the fully active dimeric GSTP1-1 has six cysteine residues titrable with DTNB. One thiol residue for monomer is masked and does not react with specific reagent, even in the presence of denaturant agents [17]. In another body of experiments, the free thiol groups of GSTP1-1 were determined before and after the reaction with captan and captafol. After the complete reaction of GSTP1-1 with pesticides at a 1:1 pesticide:GSTP1-1 molar ratio, only one cysteine residue per monomer was titrable with DTNB (Fig. 5). The incubation of the pesticide-inactivated GSTP1-1 with 100 mM DTT restored the original enzyme activity, and three cysteine residues per monomer again became titrable with DTNB (Fig. 5). These results indicate that captan and captafol may inactivate

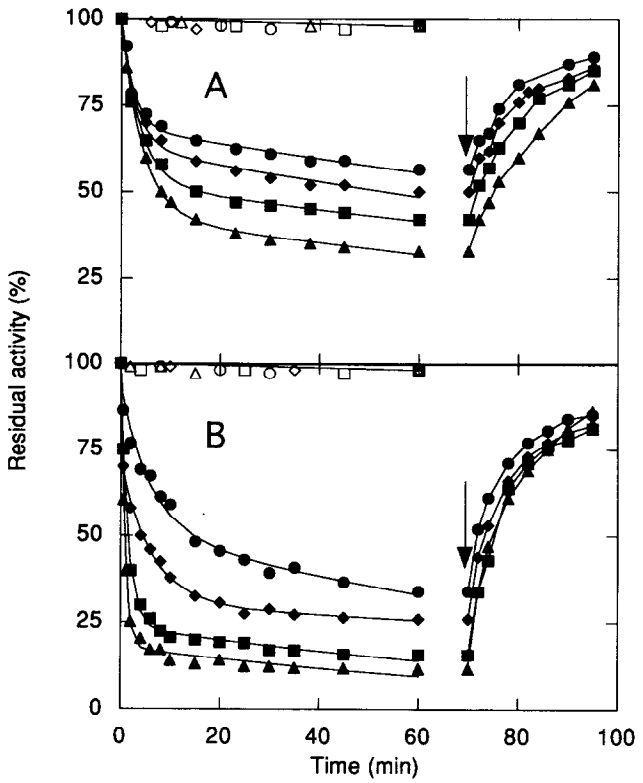

FIG. 3. Time-course of GSTP1-1 inactivation by captan (panel A) and captafol (panel B) at different pH values. About 15 μM of GSTP1-1 was incubated with pesticides (1:1 molar ratio) at 25°C for 60 min. ▲, pH 7.0; ■, pH 6.5; ◆, pH 5.8; ●, pH 5.0. The open symbols refer to the corresponding control cuvettes. At the intervals of time, adequate volumes were withdrawn and pipetted into the standard assay mixture for residual activity measurement. After removing pesticides and their byproducts with G-25 Sephadex (Pharmacia NAP-5 column), 100 mM of dithiothreitol was added (arrow).

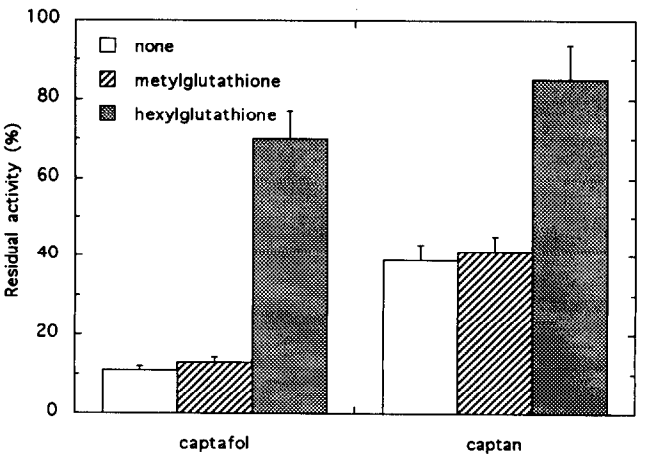

FIG. 4. Effect of methylglutathione and hexylglutathione on time-course inactivation of GSTP1-1 by captan and captafol. The experiment was carried out in 0.01 M potassium phosphate buffer pH 7.0. Except for the presence of 3 mM of methylglutathione or 3 mM of hexylglutathione, the incubation system is identical to that described in the legend of Fig. 3. Residual activity is given after 20 min of incubation.

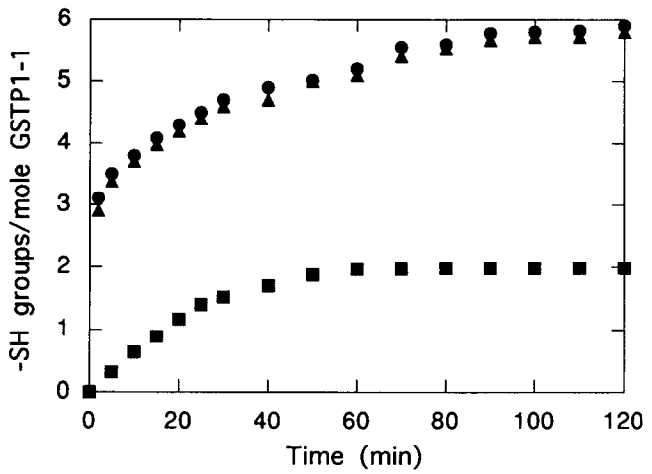

FIG. 5. Thiol group titration of GSTP1-1. GSTP1-1 (2 μM) reacted with 0.1 M DTNB at pH 8.0 before pesticide treatment (●), after pesticide treatment (■), and after dithiothreitol treatment (▲).

GSTP1-1 *via* a redox process that induces the disappearance of at least two cysteine residues per monomer. It is well established that GSTP1-1 possesses a highly reactive cysteine residue (i.e., cys47) the modification of which results in enzyme inactivation [30–33]. Cys47, which is present as thiolate ion in the protein [28], is not a structural part of the active site [10, 12] and is non-essential for catalytic activity [15, 16]. The modification of cys47 inactivates the enzyme either by conformational changes or by preventing substrate binding [30–33]. The cysteine residues of GSTP1-1 may also undergo reversible oxidative inactivation through the formation of intra- or intersubunit disulphide bonds between cys47 and cys101 [17, 18]. To better understand the role of these cysteine residues in enzyme inactivation by pesticides, the cys47ala, cys101ala, and cys47ala/cys101ala GSTP1-1 mutants were prepared and subjected to captan and captafol incubation. Cys47ala, cys101ala, and cys47ala/cys101ala GST mutants were all inactivated with increasing concentrations of captan and captafol. The time-course of inactivation of the three GSTP1-1 mutants by captan and captafol is depicted in Fig. 6. It can be seen that the GSTP1-1 and cys101ala GST mutants are more susceptible to pesticide inactivation than the cys47ala and cys47ala/cys101ala GST mutants, indicating that cys47 remains the primary target in the inactivation process. However, as indicated by the experiments with the double mutant, in addition to cys47 and cys101 residues, the other two cysteine residues present in the GSTP1-1 subunit (cys14 and cys169) may be involved in the pesticide inactivation. It has been previously demonstrated that SDS/PAGE is a useful technique to highlight the formation of intra- and intersubunit disulfide bonds; indeed, the oxidized forms of GST exhibit altered mobility on SDS/PAGE [17, 18]. When both the GSTP1-1 and GSTP1-1 mutants were treated with captan or captafol at different pesticide:enzyme molar ratios, and analyzed by SDS/PAGE under nonreducing conditions, the results in Fig. 7 were obtained. GSTP1-1 was characterized by several extra bands with an apparent molecular mass of 19.5 kDa, 21.2 kDa, 37 kDa, 38 kDa, 42.5 kDa, and 51.5 kDa, in addition to the native GSTP1-1 subunit band with a molecular mass of 23.5 kDa. These extra bands returned to the

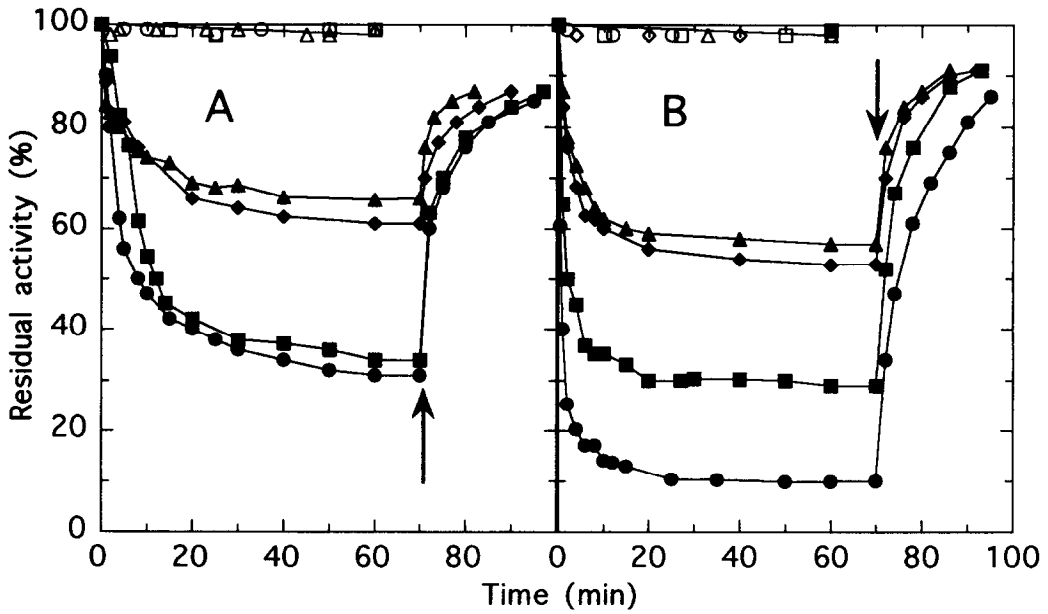

FIG. 6. Time-course of GSTP1-1 cys/ala mutant inactivation by captan (panel A) and captafol (panel B). About 15 μM of the GSTP1-1 mutant was incubated with pesticide (1:1 molar ratio) at 25°C for 60 min in 0.01 M potassium phosphate buffer pH 7.0. At specific intervals of time, adequate volumes were withdrawn and pipetted into the standard assay mixture for residual activity measurement. The arrows indicate the time of addition of 100 mM of dithiothreitol in the incubation mixtures. (●) GSTP1-1 control; (♦) Cys47ala GST mutant; (■) cys101ala GST mutant; (▲) cys47ala/cys101ala GST mutant.

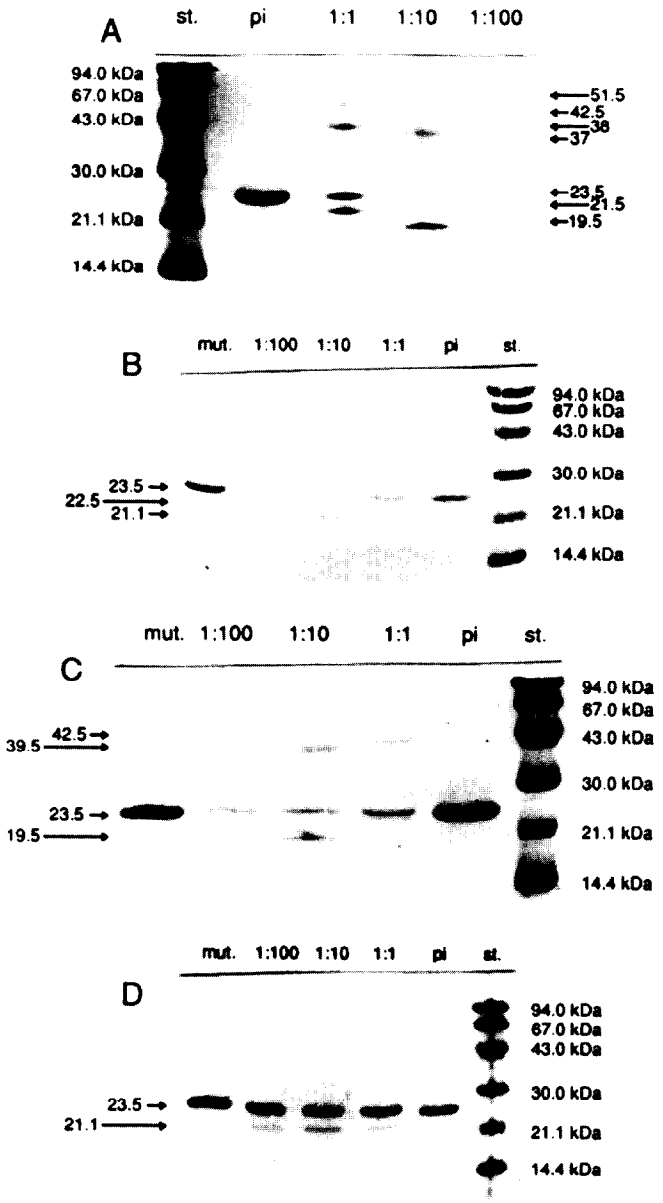

FIG. 7. SDS-PAGE of GSTP1-1 (panel A) cys47ala GST mutant (panel B), cys101ala GST mutant (Panel C), cys47ala/cys101ala GST mutant (panel D) treated with captan at 1:1, 1:10, and 1:100 enzyme:pesticide molar ratios, under nonreducing conditions. st., standard proteins from top to bottom phosphorylase b (94.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and (14.4 kDa); pi, GSTP1-1 control; mut., GSTP1-1 mutant control. On each lane, about 30 μg of protein was loaded.

native 23.5 kDa band, with concomitant restoration of activity, after treatment with dithiothreitol, confirming that they are due to disulfide bond formation. The presence on SDS/PAGE of bands with molecular masses higher and lower than 23.5 kDa indicates that both intra- and intersubunit disulfide bonds have been formed. Extra bands were also observed with the cys47ala mutant (21.2 kDa and 22.5 kDa), cys101ala (19.5 kDa, 39.5 kDa, 42.5 kDa) and the cys47ala/cys101ala mutant (21 kDa). Thus, in addition to Cys47 and cys101, the other two cysteine residues of GSTP1-1, cys14 and cys169, are involved in pesticide inactivation. In fact, the single band present in the SDS/PAGE of the double cys47ala/cys101ala mutant is certainly due to an intrasubunit disulfide bond between cys14 and cys169. It should be noted that the SDS/PAGE obtained with the cys47ala GSTP1-1 and cys47ala/cys101ala GSTP1-1 mutants are devoid of extra bands with an apparent molecular mass higher than 23.5 kDa. This means that cys47 is certainly involved in the inter-subunit disulfide bond produced by pesticide inactivation.

In summary, evidence is presented indicating that captan and captafol inactivate GSTP1-1 acting as an oxidizing agent of the isoenzyme sulphydryl groups. In particular, captan and captafol induce the formation of both intra- and intersubunit disulfide bridges involving all four cysteine residues of the GSTP1-1 subunit.

GSTP1-1 has been implicated in the multidrug resistance mechanism of some human tumor cells [4]. Selective modification of GSTP1-1 by compounds such as studied herein could represent a possible means of overcoming the resistance of tumor cells to cytostatic drugs.

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