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1521-6543/99 \$12.00 + .00

Original Research Article

Thiolation of Low- M_r Phosphotyrosine Protein Phosphatase by Thiol-Disulfides

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Summary

Thiol-disulfides cause a time- and a concentration-dependent inactivation of the low- M_r phosphotyrosine protein phosphatase (PTP). We demonstrated that six of eight enzyme cysteines have similar reactivity against 5,5'-dithiobis(nitrobenzoic acid): Their thiolation is accompanied by enzyme inactivation. The inactivation of the enzyme by glutathione disulfide also is accompanied by the thiolation of six cysteine residues. Inorganic phosphate, a competitive enzyme inhibitor, protects the enzyme from inactivation, indicating that the inactivation results from thiolation of the essential active-site cysteine of the enzyme. The inactivation is reversed by dithiothreitol. Although all PTPs have three-dimensional active-site structures very similar to each other and also have identical reaction mechanisms, the thiol group contained in the active site of low- M_r PTP seems to have lower reactivity than that of other PTPs in the protein thiolation reaction.

IUBMB *Life*, 48: 505–511, 1999

Keywords Disulfide; glutathione; phosphotyrosine protein phosphatase; PTP.

INTRODUCTION

Studies of the molecular mechanisms of signal transduction in the activation of cell growth and other important cellular processes have highlighted the critical roles elicited by protein tyrosine kinases (PTKs)¹ and phosphoprotein tyrosine phosphatases (PTPs). PTK receptors are activated by growth factors and then become phosphorylated in tyrosine residues. This initiates intracellular signaling, mainly through the recruitment of proteins containing Src homology domain 2 (SH2), which induces phos-

phorylation cascades that propagate the signal to the nucleus. The activation of several transcription factors elicits the transcription of early genes, with the successive induction of other events that lead to mitosis.

Although the main focus of most laboratories has been on the PTKs, it is generally acknowledged that PTPs are very important in the propagation, coordination, and termination of signaling cascades (1–4).

The PTP family is subdivided into four subfamilies: the “classical” PTPs, the VH1-like PTPs, the cdc25 PTPs, and the low-molecular-weight PTPs (LMW-PTP). All PTPs have the conserved active-site motif CXXXXXR and an identical catalytic mechanism (5), and both Cys and Arg are essential for activity. The Arg residue of the motif participates in the substrate binding, whereas the Cys residue performs the nucleophilic attack at the phosphorus atom of the substrate, forming an enzyme–phosphate covalent intermediate, the hydrolysis of which is the limiting step of the catalytic process. The “classical” PTPs are the largest subfamily, containing all the transmembrane enzymes as well as several intracellular related enzymes having a relatively conserved catalytic domain of ~250 residues and a strict specificity for phospho-tyrosine. Some members of this family contain SH2-domain(s) and are then recruited by tyrosine-phosphorylated proteins during signaling. The majority of VH1-like PTPs family members are able to dephosphorylate both tyrosine and threonine residues (dual-specific phosphatases). The cdc25 PTPs are involved in controlling the entry of cells into mitosis, and the LMW-PTPs seem to be involved in the down-regulation of some PTK receptors, such as the platelet-derived growth factor and insulin receptors (6–8).

Although LMW-PTP consists of a single enzyme domain without any other control domain, we have recently demonstrated that its activity can be modulated by the transient chemical modification of its active-site cysteine. In fact, both NO and H₂O₂ induce in vitro inactivation of this enzyme and the formation of a disulfide bridge between active-site Cys12 and Cys17 (9, 10). The formation of this bridge has been reported only

Received 24 May 1999; accepted 6 July 1999.

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¹Abbreviations: DTNB, 5,5'-dithiobis(nitrobenzoic acid); GSSG, glutathione disulfide; LMW-PTP, low- M_r phosphotyrosine protein phosphatase; PTK, protein tyrosine kinase; PTP, phosphotyrosine protein phosphatase; SH2, Src homology 2.

for the LMW-PTP, because this is the only PTP containing two vicinal thiols within the active-site sequence CXXXXCR. The inactivation is reverted by low- M_r thiols. The specific inactivation of LMW-PTP by NO has also been demonstrated *in vivo*, stimulating RAW 264.7 macrophages to produce NO with a bacterial lipopolysaccharide and interferon- γ (11). In addition, we have demonstrated that physiological concentrations of H_2O_2 induce inactivation, suggesting that the activity of this enzyme is modulated during oxidative stress and other physiologically relevant processes (10). Recently, Lee et al. (12) demonstrated that another PTP (PTP 1B) is transiently inactivated by H_2O_2 produced *in vivo* during cellular activation by epidermal growth factor. The reaction between LMW-PTP and NO or H_2O_2 is highly specific, because of the eight cysteines contained in the enzyme, only active-site Cys12 and Cys17 are modified (10). Other PTPs also react specifically with H_2O_2 ; that is, only the Cys contained in the active-site motif CXXXXXR is modified (13). The basis for the observed specificity is certainly the unusual reactivity of the cysteine thiol of the active-site motif. Pregel and Storer demonstrated that the titration of the PTP SHP-1 at pH 5.0 with 5,5'-dithiobis(nitrobenzoic acid) (DTNB) is a biphasic process characterised by a rapid reaction of the active-site cysteine followed by the slow reaction of the other cysteines of the molecules (14). The initial burst phase is due to the fast reaction of the active-site cysteine with DTNB.

In this paper we deal with the reaction mechanism of glutathione disulfide (GSSG) and DTNB with the LMW-PTP, which also contains within its active site a cysteine with a low pK_a value (15).

EXPERIMENTAL PROCEDURES

Human recombinant LMW-PTPs (IF2 isoenzyme) were prepared as previously described (16). All other reagents were the purest commercially available.

PTP activity was assayed as previously described, by using *p*-nitrophenyl phosphate as substrate at pH 5.3 and 37°C (9). All initial rate measurements were carried out at least in duplicate.

Protein concentration was assayed by the bicinchoninic acid method (BCA-kit) purchased from Sigma.

Modification of LMW-PTP with Glutathione Disulfide (GSSG) and with DTNB. In the experiments with GSSG, the incubation mixture (150 μ l) contained 0.01 M Tris-HCl buffer, pH 7.5, 13 μ M enzyme, and GSSG ranging from 2 to 10 mM (final concentrations). In the experiments with DTNB, the incubation mixtures (1 ml) contained 0.1 M Tris-HCl buffer, pH 7.5, 5 μ M enzyme, and 0.8 mM DTNB. The progress of the reaction was continuously recorded at 410 nm (the ϵ value for nitrobenzoate anion of 13,600 $M^{-1} cm^{-1}$ at pH 7.5 and 11,860 $M^{-1} cm^{-1}$ at pH 5.0 [14]) were used. Similar experiments were performed in 0.1 M sodium acetate buffer, pH 5.0. All experiments were performed at 25°C. Aliquots were removed at different time intervals, and the residual activity was immediately assayed. In protection experiments with inorganic phosphate, the ligand was added prior to the addition of GSSG. The number of cysteines

modified by DTNB per enzyme molecule at various inactivation levels was determined by following the time-course of both the decrease of enzyme activity and the increase of absorbance at 410 nm.

HPLC-Electrospray-Mass Spectrometry. HPLC was performed with a Vydac Peptide and Protein C₁₈ column (4.6 \times 250 mm, 5 μ m particle size) at a flow rate of 0.8 ml/min. The column was eluted with solvent A (10 mM trifluoroacetic acid in water) and solvent B (10 mM trifluoroacetic acid in acetonitrile) in the following elution gradient 0–20 min, 10–60% solvent B. The column flux was continuously introduced into the electrospray source of the mass spectrometer, and mass spectra were acquired. The mass spectrometer was the Hewlett-Packard (Palo Alto, CA) 1100 MSD mass spectrometer. The electrospray mass spectra of LMW-PTPs were obtained under the following conditions: electrospray capillary voltage, 4 kV; fragmentor voltage, 120 V; nebulizer gas (N_2), 276 kPa; drying gas (N_2), 10 l/min at 300°C; scan range, 500–2000 m/z ; scan rate, 3 ms/amu; resolution, > 1 amu.

RESULTS

Fig. 1A shows that GSSG causes the time- and concentration-dependent inactivation of the LMW-PTP. The reaction seems to proceed at a relatively slow rate at 25°C and pH 7.5; ~90% inactivation is produced after 60 min of enzyme incubation with 10 mM GSSG. The enzyme was protected from inactivation by P_i (Fig. 1B), a well-known competitive inhibitor of the enzyme, suggesting that the active-site cysteine of LMW-PTP participates in the thiol–disulfide interchange reaction. Furthermore, we have demonstrated that the inactivation is reversed by dithiothreitol (Fig. 1A).

We used combined HPLC–electrospray–mass spectrometry to determine the relationship between the number of cysteines modified by GSSG in the enzyme molecule and the residual activity. Fig. 2 shows the reversed-phase HPLC profiles at reaction times of 0, 3, 12, and 40 min, revealing that additional peaks appear during the reaction between LMW-PTP and GSSG. The electrospray–mass spectrometric analyses performed on these peaks are reported in Fig. 3, which shows that the progress of enzyme inactivation is accompanied by the formation of mixed disulfides between enzyme and glutathione as follows: at 21% inactivation both mono and di-thiolated enzyme derivatives were present, whereas at 59% inactivation the di- and tri-thiolated enzyme species were detected. At 88% inactivation the mass analyses demonstrated mainly the presence of enzyme–glutathione derivatives, in which an enzyme molecule was linked with four, five, and six glutathione molecules. These findings demonstrate that the reaction of LMW-PTP with GSSG, which leads to enzyme inactivation, is not exclusively directed at the cysteine contained in the active site. Several additional cysteines react simultaneously with this reagent, but inactivation is probably due to the modification of the essential active-site cysteine. These results are unexpected, because other relatively nonspecific reagents (such as iodoacetate, nitric oxide, and hydrogen

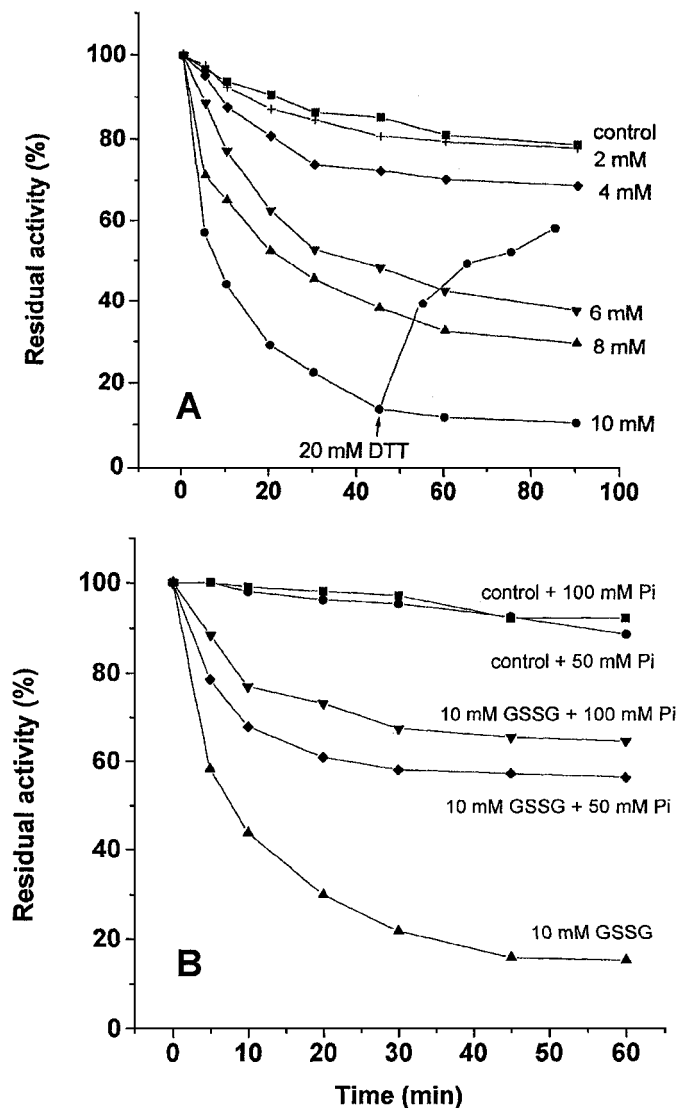


Figure 1. Inactivation of LMW-PTP by glutathione disulfide. (A) The enzyme ($13 \mu\text{M}$, $150 \mu\text{l}$ final volume) was treated with 2, 4, 6, 8, and 10 mM GSSG (final concentrations). The incubation was performed at 25°C in 0.1 M Tris-HCl buffer, pH 7.5. At various time intervals, aliquots were removed and assayed for residual enzyme activity. The arrow indicates the time of addition of dithiothreitol (DTT) in the reactivation experiment. (B) The protection elicited by P_i , a competitive inhibitor, against the enzyme inactivation by GSSG.

peroxide) that inactivate LMW-PTP react specifically with the cysteine contained in the active site, even though the enzyme contains eight cysteine sulfhydryl groups per molecule (17). Pregel and Storer (14) demonstrated that DTNB (an analog of GSSG with respect to thiol-disulfide exchange reactions) also elicits high reactivity against the cysteine contained in the PTP active site. They reported that at pH 5.0 and 25°C the reaction between the SHP-1 PTP and DTNB proceeds through an

initial burst phase followed by a slower secondary phase, reflecting the unusually low pK_a value of the essential active-site cysteine of PTPs (14, 15). Because LMW-PTP also contains this particular cysteine located in a similar environment (15), we performed LMW-PTP inactivation experiments with DTNB. Fig. 4A reports the time-courses of the reactions of LMW-PTP with DTNB at 25°C , both at pH 5.0 and pH 7.5; the inset of Fig. 4A shows the LMW-PTP inactivation time-courses at both pH values. No burst reaction phases were observed at either pH value. However, the reaction between LMW-PTP and DTNB is pH-dependent, indicating that the thiolate form is the most reactive one. Fig. 4B reports the plot of remaining activity versus the number of cysteines modified per enzyme molecule at pH 5.0 during inactivation by DTNB. For inactivation as great as 85%, the experimental points follow a straight line that intersects the abscissa at a point corresponding to the modification of six cysteine residues per enzyme molecule. After that, additional cysteines are modified without further loss of activity. Because the enzyme contains eight cysteine residues per molecule, our findings clearly indicate that at pH 5.0 two classes of cysteines possessing different reactivities are contained in the enzyme. In contrast, the plot of residual activity at pH 7.5 against modified cysteines per enzyme molecule appears monophasic (Fig. 4C), indicating that at this pH value all cysteines possess the same reactivity against DTNB.

DISCUSSION

The LMW-PTPs, which contain members present in a wide variety of organisms, including bacteria, yeast, and animals, is a subfamily of the large PTP family, a family involved in several important cellular signaling processes (5). In vertebrates, LMW-PTPs participate in the complex signaling cascades originating from the stimulation of some growth factor receptors by their respective specific factors. In particular, we have demonstrated that the enzyme is able to bind and dephosphorylate PDGF and insulin receptors only when they are activated, thereby down-regulating them (6, 8, 18, 19). In addition, the transfection of the active enzyme and of its negative-dominant form (the mutant C12S, which is able to bind substrates but is catalytically inactive) in NIH/3T3 fibroblasts demonstrated that, whereas the growth rate of these cells is decreased by overexpression of the active enzyme, it is increased by the overexpression of the negative-dominant form (18). These phenotype behaviours seem to be mainly determined by the LMW-PTP action in the G_0 phase of the cell cycle, although the involvement of this enzyme in other cell cycle phases cannot be excluded (7).

The LMW-PTP gene has remained highly conserved throughout evolution from yeast to humans. The homologs from *Saccharomyces cerevisiae* (Ltp1) (20) and *Schizosaccharomyces pombe* (Stp1) (21) are respectively 39% and 42% identical to the mammalian enzyme. In microorganisms the enzyme performs different roles from those performed in animals. Bugert and Geider (22) have suggested that the enzyme, which is codified by the

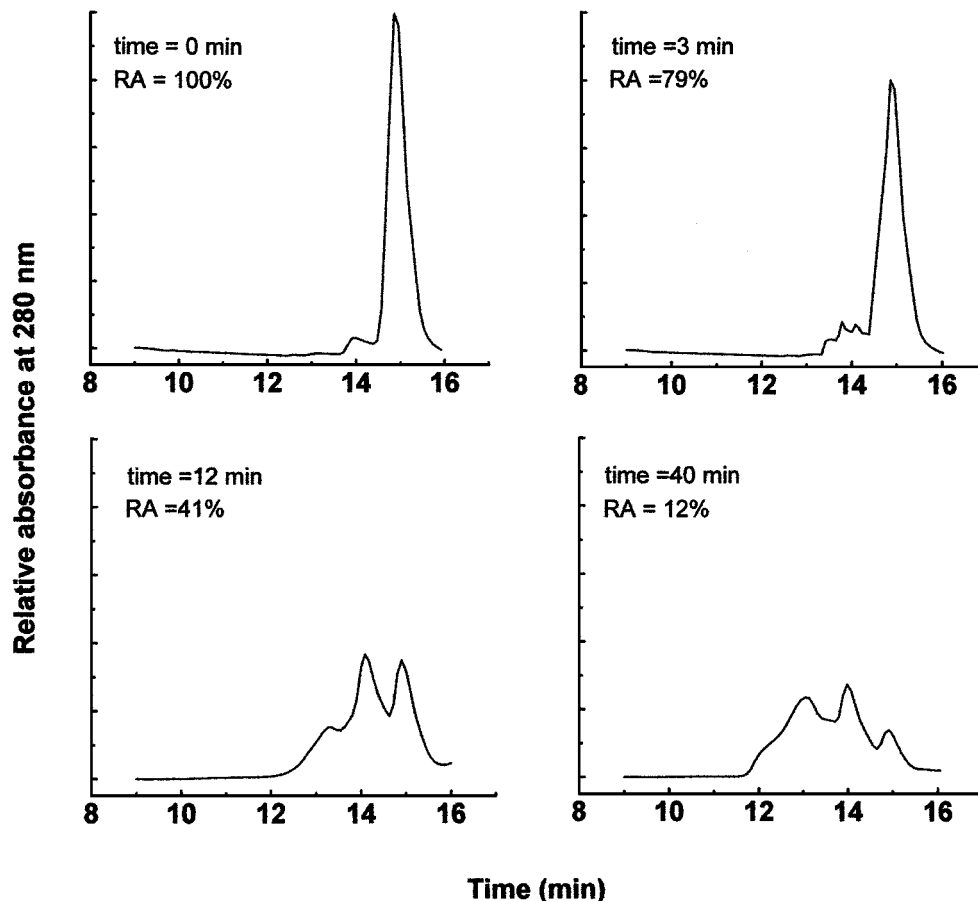


Figure 2. LMW-PTP inactivation by GSSG: time-course HPLC analyses. At the indicated times, samples from the enzyme-GSSG mixture were injected into the HPLC column (Altex, Ultrapore RPSC, 4.6×75 mm, $5 \mu\text{m}$). Elution was done with solvent A (10 mM trifluoroacetic acid in water) and solvent B (10 mM trifluoroacetic acid in acetonitrile) at the gradient of 0–20 min, 10–60% solvent B. The flow rate was 0.8 ml/min. The modified enzyme forms eluted between 12.5 and 14.5 min, whereas the nonmodified enzyme eluted at ~ 15 min. RA, residual activity.

ams region responsible for amylovoran synthesis in the fireblight pathogen *Erwinia amylovora*, participates in the synthesis of this exopolysaccharide, essential for the pathogenesis of some rosaceous plants. In addition, Grangeasse et al. (23) have reported that the homologous gene from *Acinetobacter johnsonii* is able to dephosphorylate a novel bacterial PTK that autophosphorylates on tyrosine. Thus, this very interesting enzyme is likely to be involved in the modulation of the activity of an ancient PTK, and perhaps participates in the regulation of a fundamental bacterial function. We have previously demonstrated that this small enzyme, which consists of a single catalytic domain without any regulatory domain, is regulated by the chemical modification of the active-site cysteine by physiological compounds. Hydrogen peroxide (10) and superoxide anion (24), both produced during oxidative stress conditions, as well as nitric oxide (9), are able to react specifically with the active-site cysteine, whereas other cysteines in the enzyme molecule (the total is eight per molecule) remain unmodified.

Several cell conditions determine the alteration in the normal (GSSG)/(GSH) redox ratio. Severe oxidative insults, such as the one occurring during phagocytosis in leukocytes (25), drastically modify cellular redox status and also activate glutathione peroxidase action, thus enhancing the concentration of GSSG. This event may cause the formation of mixed disulfides in proteins, particularly those having highly reactive thiol groups (26, 27). This particular modification is transient, because GSSG is reduced back to GSH by the action of the NADPH-dependent glutathione reductase, thus setting up redox conditions that reverse protein thiolation. We have tested GSSG to determine whether the active-site cysteine of the enzyme produces a specific reaction with this metabolite. The possible specificity of the protein thiolation reaction was suggested by Pregel and Storer (14), who found that the cysteine contained in the conserved active-site motif CXXXXXR of SHP-1 PTP reacted with DTNB faster than other cysteines contained in this PTP molecule (with this PTP, the reaction proceeded through an initial burst phase followed

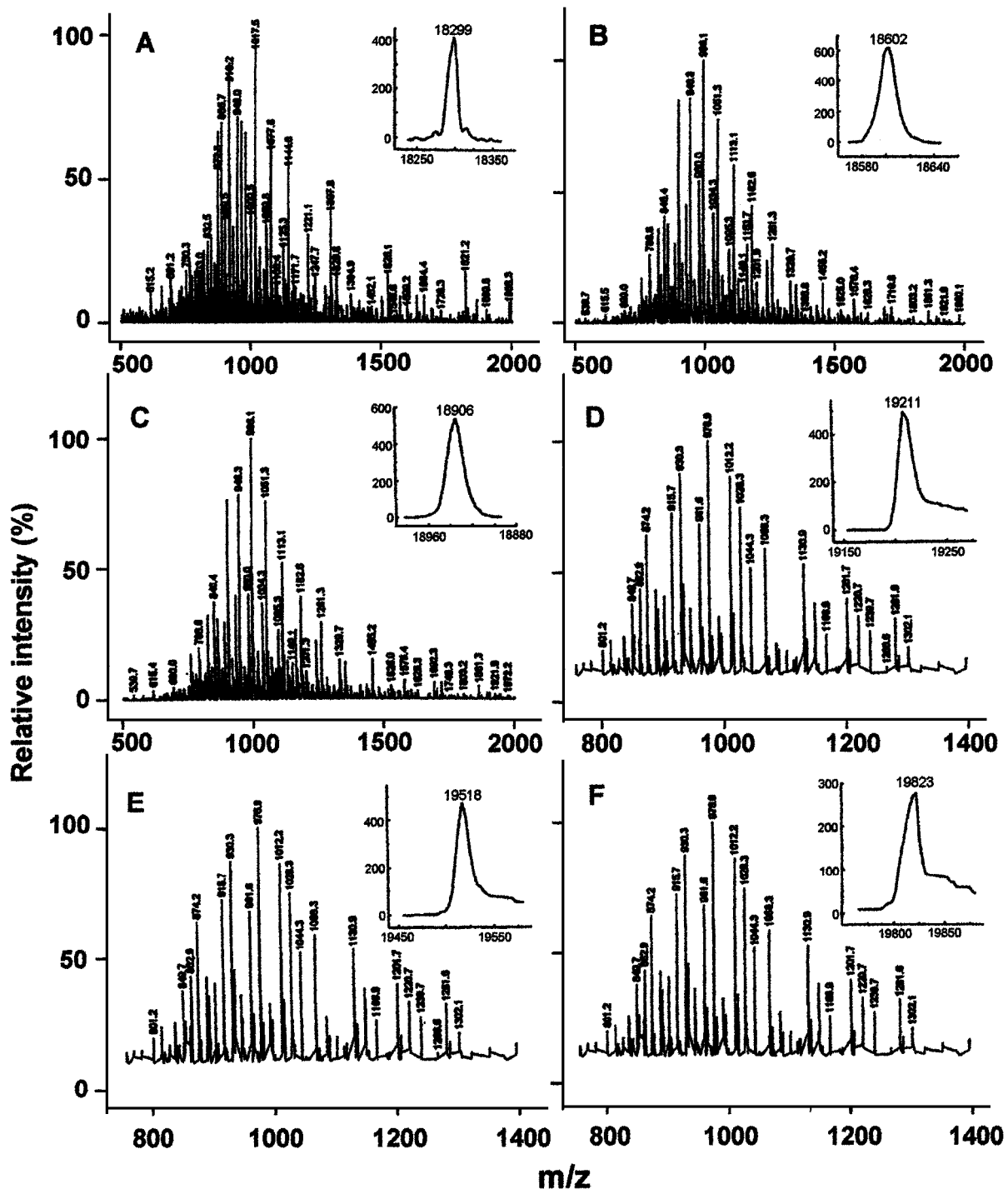


Figure 3. Electro spray-mass spectra of thiolated LMW-PTP forms. The flux from the HPLC column (Fig. 2) was continuously introduced into the electro spray source. (A–F) Spectra of mono-, di-, tri-, tetra-, penta-, and hexa-thiolated molecular forms of the enzyme, respectively. All spectra were taken in the 12.5–14.5-min zone of the HPLC chromatograms (Fig. 2), which contains all modified enzyme forms. The special mass spectrometry computer program (Hewlett-Packard) selected the various families of peaks possessing the appropriate charges useful to calculate the reconstructed mass spectra (see the inset in each panel). The electro spray mass spectrum of the nonmodified enzyme was also acquired (not reported). We found a mass value of 17,990, which is very close to the molecular mass calculated from the amino acid sequence of LMW-PTP (17,990.3 Da).

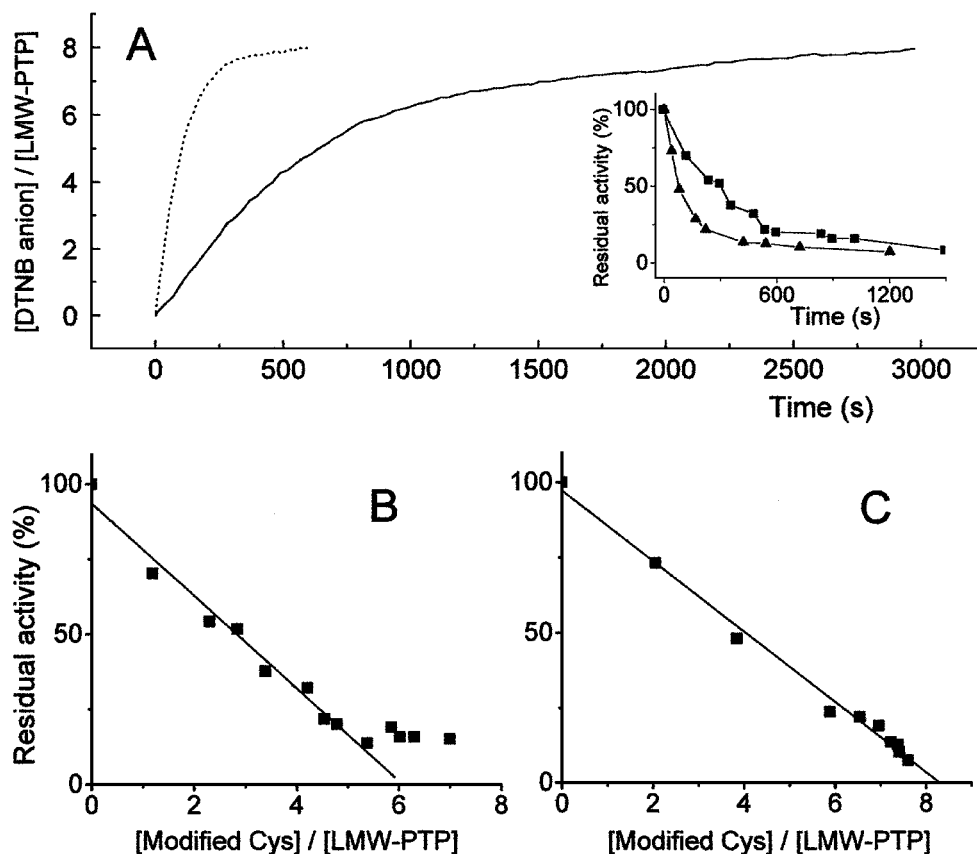


Figure 4. Inactivation and stoichiometry of the modification of LMW-PTP by DTNB. (A) Time courses of protein cysteine modification by DTNB. The incubation mixtures (1 ml, 0.1 M Tris-HCl buffer, pH 7.5) contained 5 μ M enzyme and 0.8 mM DTNB (dotted line). Similar experiments were performed in 0.1 M sodium acetate buffer, pH 5.0 (continuous line). The reaction progress was continuously recorded at 410 nm: the absorbance data were converted into moles of released thio-nitrobenzoate anion by use of a computer program. All experiments were performed at 25°C. Aliquots were removed at different time intervals, and the residual activity was immediately assayed. (A, inset) Inactivation time courses at pH 7.5 (\blacktriangle) and pH 5.0 (\blacksquare). (B) Residual activity at pH 5.0 plotted against moles of modified residues per mole of enzyme. (C) Similar to B, but with measurements performed at pH 7.5.

by a slower secondary phase). In contrast, our findings clearly demonstrate that LMW-PTP is unable to give specific thiolation reactions with both GSSG and DTNB, suggesting that this type of reaction is not involved in the regulation of LMW-PTP activity *in vivo*. Moreover, the kinetics of the reaction of LMW-PTP with glutathione disulfide seems too slow to have physiological relevance. In fact, cellular GSSG is reduced by glutathione reductase, which rapidly rescues the normal highly reduced cellular environment. All our findings demonstrate that six of the eight cysteines in LMW-PTP possess similar reactivity against thiol-disulfides, but inactivation is probably attributable to the chemical modification of only one of these, i.e., the essential active-site cysteine. In fact P_i , which is a competitive enzyme inhibitor and then specifically interacts with the enzyme's active site, is able to protect the enzyme from inactivation. The present findings contrast with those that have demonstrated very specific reactions between the LMW-PTP active-site cysteine and

other reactive molecules, such as iodoacetate (17), hydrogen peroxide (10), and nitric oxide (9). Nevertheless, our findings on LMW-PTP thiolation by thiol-disulfides do not exclude that this reaction has a role in the regulation of other PTPs, particularly of those behaving like SHP-1, i.e., containing a cysteine residue that is very reactive against thiol-disulfides (14).

Although all PTPs have very similar three-dimensional active-site structures as well as identical reaction mechanisms, the thiol group contained in the active site of LMW-PTP seems to have lower reactivity than that of other PTPs in the thiolation reaction occurring between disulfides and the enzyme.

ACKNOWLEDGEMENTS

This work was supported in part by CNR (grant no. 97.03810. CT14, and Target Project on Biotechnology), in part by MURST (1997), "Meccanismi biochimici di controllo delle funzioni

cellulari,” and in part by the Italian Association for Cancer Research (AIRC). We thank the Centro Interdipartimentale di Servizi di Spettrometria di Massa, Università di Firenze, for the acquisition and elaboration of electrospray–mass spectra.

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