The Inactivation Mechanism of Low Molecular Weight Phosphotyrosine-protein Phosphatase by H$_2$O$_2$*

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Low molecular weight phosphotyrosine-protein phosphatase (LMW-PTP) shares no general sequence homology with other PTPs, although it has an active site sequence motif CXXXXXR and a reaction mechanism identical to those of all PTPs. The main function of this enzyme is the down-regulation of platelet-derived growth factor and insulin receptors. Both human LMW-PTP isoenzymes are inactivated by H$_2$O$_2$. The enzymes are protected from inactivation by P$_i$, a competitive inhibitor, suggesting that the H$_2$O$_2$ reaction is directed to active site. Analysis of free thiols performed on the inactivated enzymes demonstrates that only two out of the eight LMW-PTP cysteines are modified. Time-course high performance liquid chromatography-electrospray mass spectrometry, together with specific radiolabeling and tryptic fingerprint analyses, enables us to demonstrate that H$_2$O$_2$ causes the oxidation of Cys-12 and Cys-17 to form a disulfide bond. Because both residues are localized into the active site region, this modification inactivates the enzyme. Fluorescence spectroscopy experiments suggest that the fold of the enzyme is modified during oxidation by H$_2$O$_2$. Because a physiological concentration of H$_2$O$_2$ produces enzyme inactivation and considering that the activity is restored by reduction with low molecular weight thiols, we suggest that oxidative stress conditions and other processes producing hydrogen peroxide regulate the LMW-PTP in the cell.

Protein tyrosine phosphorylation in eucaryotes is a key mechanism for cellular control, because it is involved in several processes, such as cellular metabolism, proliferation, differentiation, and oncogenic transformation (1). A fine balancing of cellular protein tyrosine phosphorylation levels is determined by regulating the activities of protein-tyrosine kinases and/or protein-tyrosine phosphatases (PTPs).1 Receptor protein-tyrosine kinases are considered to be the major enzymes regulating mitogenic protein phosphorylation cascades; nevertheless, the presence of SH2 domains in particular PTPs and the receptor-like structure of some membrane PTPs clearly indicate that PTPs are also regulated in the cell.

The PTP superfamily consists of four main families: the tyrosine-specific phosphatases, the VH1-like dual specificity phosphatases, the cdc25 phosphatases, and the low molecular weight phosphatases (LMW-PTPs). Despite extremely limited sequence similarity, all share an active site motif consisting of a cysteine and an arginine separated by five residues (CXXXXXR, where X is any amino acid). All PTPs have identical catalytic mechanism, which involves the formation of a cysteinyl-phosphate intermediate (2).

Recent papers from our laboratory have demonstrated that LMW-PTP is involved in the regulation of cellular signaling started by the activation of PDGF and insulin receptors (3–5). In fact, the overexpression of the wild type enzyme in NIH/3T3 cells causes decrease of cellular growth rate and of phosphorylation level of the PDGF receptor (3). Furthermore, the overexpression in NIH/3T3 cells of a dominant negative mutant (C12S), which is still able to bind substrates but is catalytically inactive, has opposite effect (6). In addition, immunoprecipitation experiments (using anti-PDGF receptor, anti-insulin receptor, and anti-LMW-PTP antibodies) performed with cells overexpressing the dominant negative enzyme form have demonstrated that the enzyme associates with these receptors only when they are stimulated by their specific growth factors (5, 6).

Furthermore, studies performed on the mitogenic transduction pathways starting from tyrosine kinase receptors demonstrated that LMW-PTP selectively interferes with the Src and the signal transducer and activator of transcription pathways in PDGF downstream signaling (4) and the Src pathway in insulin downstream signaling (5). In the case of the latter receptor, we have also demonstrated that overexpression of the dominant negative and wild type LMW-PTPs influences glycogen synthesis rate (5). Other studies have also demonstrated that LMW-PTP was phosphorylated in vitro by v-Src and by Lck kinases and that the phosphorylation caused a strong enhancement of its activity (7, 8). In addition, Tailor et al. (8) found that LMW-PTP is phosphorylated in resting T cells and that the protein rapidly loses its phosphotyrosine on stimulation of these cells. All of these findings indicate that there are complex relationships, still not adequately understood, among LMW-PTP, protein-tyrosine kinase receptors, and Src kinases. Very recently, the recruitment of LMW-PTP by ephrin-tyrosine kinase receptors has been demonstrated (9).

This paper deals with the mechanism of LMW-PTP inactivation by hydrogen peroxide, a compound endogenously produced in the cell. Significant enzyme inactivation levels are
determined by physiological concentrations of hydrogen peroxide, such as those produced during oxidative stress conditions or during signaling processes initiated by a variety of ligands including cytokines, peptide growth factors acting through tyrosine kinase, and G-protein-coupled receptors (10). This suggests that H$_2$O$_2$ regulates LMW-PTP activity in vivo.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human recombinant LMW-PTPs (IF1 and IF2 isoenzymes) were prepared as described previously (11). Iodo$^{[14]C}$acetate was purchased from NEN Life Science Products. Trypsin was purchased from Sigma. The HPLC separations were performed by a reverse-phase C4-column, 4.5 × 75 mm, 5 $\mu$m, Altex ULTRAPR column, or with a reverse-phase column, 4.5 × 250 mm, 5 $\mu$m, Vydac Peptide and Protein C18. All other reagents were the purest commercially available.

**Protein Assay and Kinetic Constants**—PTP activity was assayed as described previously, using p-nitrophenyl phosphate as substrate at pH 5.3 and 37 °C (12). The enzyme unit is defined as the amount of enzyme that catalyzes the hydrolysis of 1 nmol of p-nitrophenol phosphate per min under the described conditions. The experimental data were fitted with the kinetic equations using the Fig. P program (Biosoft). All initial rate measurements were carried out at least in triplicate.

**LMW-PTP with H$_2$O$_2$—Stock solutions of H$_2$O$_2$ were titrated before use, using a standard solution of KMnO$_4$. The modification reactions were performed at 25 °C in 0.1 M Tris-HCl buffer, pH 7.5. The incubation mixture (300 $\mu$L) contained 10 $\mu$g of LMW-PTP (IF1 or IF2 isoform) and varying H$_2$O$_2$ concentrations (0.01–25.0 mM). Aliquots were withdrawn at different time intervals, and the residual activity was immediately assayed. In protection experiments with inorganic phosphate, a well known LMW-PTP inhibitor, the ligand was added prior to the addition of the H$_2$O$_2$ stock solution.

**Determination of Free Thiol Groups**—Free thiol groups were titrated by 5-(octyldithio)-2-nitrobenzoate as described by Faulstich et al. (13). This reagent is able to react with protein thiols faster than Ellman reagent, because it contains a lipophilic carbon chain and no negative charges. The reagent easily diffuses in the protein core and thus it rapidly reacts with all protein thiols. Free thiols were titrated before use, using a standard solution of KMnO$_4$. The modification reactions were performed at 25 °C in 0.1 M Tris-HCl buffer, pH 7.5. The incubation mixture (300 $\mu$L) contained 10 $\mu$g of LMW-PTP (IF1 or IF2 isoform) and varying H$_2$O$_2$ concentrations (0.01–25.0 mM). Aliquots were withdrawn at different time intervals, and the residual activity was immediately assayed. In protection experiments with inorganic phosphate, a well known LMW-PTP inhibitor, the ligand was added prior to the addition of the H$_2$O$_2$ stock solution.

**Enzyme Assay**—Enzymic activity was measured by the kinetic colorimetric assay of Ellman (14). The chromophore formed by the reaction of the Ellman reagent, because it contains a lipophilic carbon chain and no negative charges. The reagent easily diffuses in the protein core and thus it rapidly reacts with all protein thiols. Free thiols were titrated before use, using a standard solution of KMnO$_4$. The modification reactions were performed at 25 °C in 0.1 M Tris-HCl buffer, pH 7.5. The incubation mixture (300 $\mu$L) contained 10 $\mu$g of LMW-PTP (IF1 or IF2 isoform) and varying H$_2$O$_2$ concentrations (0.01–25.0 mM). Aliquots were withdrawn at different time intervals, and the residual activity was immediately assayed. In protection experiments with inorganic phosphate, a well known LMW-PTP inhibitor, the ligand was added prior to the addition of the H$_2$O$_2$ stock solution.

**Protein Determination**—Protein concentration was assayed by the BCA kit, purchased from Sigma.

**RESULTS**

**LMW-PTP Inactivation by Hydrogen Peroxide**—Incubation of both IF1 and IF2 isoforms of LMW-PTP with H$_2$O$_2$ at pH 7.5 and 25 °C resulted in a time- and concentration-dependent loss of enzymatic activity (Fig. 1, A and B, respectively). These figures show that in the range 0.01–0.25 mM H$_2$O$_2$ concentrations, the inactivation reactions obey apparent first-order kinetics. The apparent first-order inactivation constants ($k_{obs}$) can be calculated by plotting the ln of percent residual activity versus time and fitting a linear equation. The replots of $k_{obs}$ as a function of H$_2$O$_2$ concentration (Fig. 1, A and B, insets) gave straight lines, indicating that the inactivation of LMW-PTP by H$_2$O$_2$ is a bimolecular process. The calculated second-order rate constants for the H$_2$O$_2$ inactivation of IF1 and IF2 LMW-PTP isomers are $13.1 \pm 0.2$ and $18.3 \pm 0.5$ M$^{-1}$ s$^{-1}$, respectively. Similar experiments were performed in the presence of 2 mM EDTA and gave the following second-order rate constants values: IF1 = $10.9 \pm 0.4$ M$^{-1}$ s$^{-1}$, IF2 = $13.2 \pm 0.6$ M$^{-1}$ s$^{-1}$.

**P$_i$ Protection Against H$_2$O$_2$ Inactivation of LMW-PTP and Reactivation by Thiols—Inorganic phosphate, which is a competitive inhibitor of LMW-PTP, was tested in order to determine whether or not it protects the enzyme from inactivation.

**Stoichiometry of Enzyme Modification**—Table 1 shows the titration data of free thiols performed on untreated and H$_2$O$_2$-inactivated IF1 and IF2 isoenzymes. Both untreated IF1 and IF2 LMW-PTPs contain 8 free thiols per enzyme molecule, whereas each H$_2$O$_2$-inactivated isoform contains only about 6 free thiols per enzyme molecule. These findings indicate that...
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**FIG. 2. Protection of LMW-PTP inactivation by P₁ and reactivation by thiols.** The reactivation experiments were performed by adding 10 mM dithiothreitol (DTT) at the time indicated by the arrow. The experimental conditions were the same as indicated in Fig. 1. Redrawn from ref. 13. If control; □ IF1 control; ■ IF1 + 0.25 mM H₂O₂; ● IF1 + 0.25 mM H₂O₂ + 20 mM P₁; △ IF2 + 0.25 mM H₂O₂ + 20 mM P₁.

**TABLE I**

**Stoichiometry of LMW-PTP modification by H₂O₂**

| LMW-PTP | Free thiol groups |
|---------|-------------------|
| IF1     | 8.2 (8)           |
| H₂O₂-modified IF1 | 6.1              |
| IF2     | 7.9 (8)           |
| H₂O₂-modified IF2 | 5.8              |

* Determined spectrophotometrically (13).

H₂O₂ inactivates LMW-PTPs through the oxidation of two cysteine residues.

**HPLC Electrospray Mass Spectrometry Analyses**—These analyses were performed in order to reveal the type of chemical modification of LMW-PTPs caused by H₂O₂. The left panels of Fig. 3 show the HPLC time-course analyses carried out during LMW-PTP (IF1 isoenzyme) inactivation. It can be seen that the decrease in enzyme activity due to the H₂O₂ reaction is accompanied by formation and increase of a new protein peak (peak II) having a retention time lower than that of the untreated enzyme (peak I). Fig. 4, A and C, shows the electrospray mass spectra of peaks I and II, respectively. Fig. 4, B and D, reports the charge distribution of a number of peaks selected by the computer deconvolution program to calculate the protein masses from A and C spectra (Fig. 4, B and D, insets). It can be seen that the H₂O₂-inactivated IF1-LMW-PTP (peak II; Fig. 3, left panels) has a molecular mass of 18,081 Da, which is 2 units lower than that of the untreated enzyme (molecular mass, 18,083 Da). These findings, as well as the titration of free thiols remaining in the enzyme molecule after inactivation, indicate that H₂O₂ induced the formation of a single disulfide bridge in the enzyme molecule, which contains eight cysteine residues. Identical conclusions can be drawn from the results found for the IF2-LMW-PTP isoform (Fig. 3, right panels). In this case, the electrospray mass spectra of peaks I and II gave the following results: mass of peak I, 17,990 Da; mass of peak II, 17,988 Da.

**Identification of the Site(s) Modified by H₂O₂ during LMW-PTP Inactivation**—Taking into consideration the findings reported above, we performed the following experiment in order to identify the molecular site(s) of H₂O₂ action: the IF1-LMW-PTP (50 nmol), dissolved in 1.2 ml of 0.1 M Tris-HCl buffer, pH 7.5, was incubated in the presence of 0.1 mM H₂O₂ at 25 °C. At the level of 10% residual activity, the reaction was stopped by adding 10 volumes of cold ethanol to precipitate the H₂O₂-modified enzyme. The precipitate was collected by centrifugation at 25,300 × g, washed with cold ethanol, collected again by centrifugation, and finally dissolved in 0.1 M Tris-HCl buffer, pH 8.7, containing 6 mM guanidinium chloride and 0.645 mM iodoacetate (specific radioactivity, 0.85 mCi/mmol) to label all cysteine residues oxidized by H₂O₂ during enzyme inactivation. After 15 min at room temperature, the mixture was treated with 5 μl of 2-mercaptoethanol. The mixture was incubated overnight at 40 °C in order to reduce S—S bonds formed during the H₂O₂ enzyme inactivation and then treated with 0.645 m iodo[¹⁴C]acetate (specific radioactivity, 0.85 μCi/μmol) to label all cysteine residues oxidized by H₂O₂ during enzyme inactivation. After 15 min at room temperature, the mixture was treated with 5 μl of 2-mercaptoethanol, and the labeled protein was purified from reagents by reverse phase HPLC on the ULTRAPURE RPSC column. This Cm enzyme (20 nmol) was dissolved in 100 μl of 0.1 M Tris-HCl buffer, pH 8.5, containing 6 mM guanidinium chloride and treated with 5 μl of 2-mercaptoethanol. The mixture was incubated overnight at 40 °C in order to reduce S—S bonds formed during the H₂O₂ enzyme inactivation and then treated with 0.645 m iodo[¹⁴C]acetate (specific radioactivity, 0.85 μCi/μmol) to label all cysteine residues oxidized by H₂O₂ during enzyme inactivation. After 15 min at room temperature, the mixture was treated with 5 μl of 2-mercaptoethanol, and the labeled protein was purified from reagents by reverse phase HPLC (C4-column) as described above. The enzyme peak was dried by a Savant vacuum evaporator, dissolved in 6 mM guanidinium chloride, and purified again by reverse phase HPLC (C4-column) in order to completely eliminate low molecular weight radioactive compounds.

The [¹⁴C]-labeled protein was digested with trypsin, and tryptic peptides were separated as described under “Experimental Procedures.” Fig. 5 shows the HPLC separation of tryptic peptides performed with the Vydac C18 column. All peaks were collected manually, and [¹⁴C] radioactivity was measured by liquid scintillation counting.
reported in Table II; T3 is identified as the CCR 150 trypptic peptide, whereas T37 is identified as the SVLFVCLGNICR 18 trypptic peptide (the underlined characters indicate the conserved active site motif of PTPs). Furthermore, the amino acid composition of T25 enabled us to identify it as the fragment VCLGNICR18, originating from an unexpected subfragmentation of T37 (all sequence positions refer to the natural human IF1 LMW-PTP isoform; the recombinant enzyme contains two N-terminal additional residues (Gly-Ser)). We measured the specific radioactivity of all labeled peaks. Table II shows the results: it can be seen that the stoichiometry of iodoacetate incorporation into T25 is 2.2 Cm groups/mol of peptide (near integer 5/2), whereas into T37 it is 1.9 (near integer 5/2) Cm groups/mol of peptide. These findings demonstrate that both Cys-12 and Cys-17 were completely derivatized with iodo[14C]acetate. On the other hand, the stoichiometry of modification of T3 by iodo[14C]acetate is 0.25 mol of Cm groups per mol of peptide; because T3 contains 2 Cys residues per mol of peptide, this value suggests that only a fraction of one or both Cys residues was derivatized by iodo[14C]acetate.

All of these findings, together with those obtained with the HPLC electrospray mass spectrometry analyses, demonstrate that the mechanism of H2O2 inactivation of LMW-PTP involves the formation of a disulfide bridge between Cys-12 and Cys-17, both contained in the enzyme active site.

**Fluorescence Spectroscopy Experiments**—The rather good separation between the H2O2-inactivated and active enzymes observed in the reverse phase HPLC analyses (Fig. 3) suggested to us that the formation of the disulfide bridge between...
Cys-12 and Cys-17 is accompanied (or determined) by an enzyme conformational change. In fact, the partition of proteins between the two chromatography phases depends on the protein surface properties. The hypothesis of fold modifications during H$_2$O$_2$ inactivation agrees with the x-ray crystal structure of LMW-PTP. Whereas the Cys-12 –SH group is localized at the bottom of the active site cavity (where the phosphate moiety of the substrate establishes the main interactions), the –SH group of Cys-17 is localized at a distance of about 7 Å from the Cys-12 –SH group (Fig. 6). The formation of an S= S bridge requires that the two thiol groups move from their original positions and approach each other. Thus, we performed fluorescence spectroscopy experiments to test this hypothesis.

Fig. 7A shows that the decrease of IF1 activity caused by H$_2$O$_2$ is accompanied by a red-shift of the fluorescence emission $\lambda_{\text{max}}$, whereas the quantum yield does not greatly change (the excitation wavelength was 280 nm). On the contrary, similar experiments performed with IF2 gave different results. Fig. 7C shows that the H$_2$O$_2$ inactivation of IF2 isoenzyme causes a blue-shift of the fluorescence emission $\lambda_{\text{max}}$ and a fairly strong decrease of the fluorescence quantum yield.

We also compared the spectra of H$_2$O$_2$-inactivated LMW-PTPs with those of the thermally denatured forms. Fig. 7, B and D, shows representative fluorescence emission spectra of the untreated and thermally denatured IF1 and IF2. It can be seen that the reduction of fluorescence quantum yields of both denatured proteins is stronger than that observed for H$_2$O$_2$-inactivated enzymes. Furthermore, both denatured proteins show red-shifts of the fluorescence emission maximum, indicating an exposure of Trp residues to a more polar environment.

Considering H$_2$O$_2$ inactivation, we can note that the stronger effects on fluorescence properties observed for the IF2 isoform with respect to those of IF1 could be justified taking into account that IF2 contains two Trp residues (Trp-39 and Trp-49), whereas IF1 contains Trp-39 only. Trp-49 is localized in a long loop near the active site pocket and exposed to the solvent, whereas Trp-39 is inserted into the main hydrophobic protein core (Fig. 6). We suggest that the strong decrease in the fluorescence emission quantum yield of the H$_2$O$_2$-inactivated IF2 is due to a change in the local environment of the loop containing Trp-49, with little modification of the main protein fold. This agrees with the small fluorescence differences between the untreated and H$_2$O$_2$-inactivated IF1, which does not contain Trp residues in this loop. All of these findings indicate that H$_2$O$_2$ oxidation of LMW-PTPs causes changes in the arrangement of secondary structures of the enzyme core that are limited but sufficient to favor the formation of the disulfide bridge between Cys-12 and Cys-17. We have also measured the kinetics of fluorescence modifications during H$_2$O$_2$ inactivation of both IF1 and IF2. The data fit well with first-order kinetics; in Table III, the calculated $k_{\text{obs}}$ values for the fluorescence signal reduction are compared with those calculated from H$_2$O$_2$ inactivation experiments. The $k_{\text{obs}}$ values for both IF1 and IF2 inactivation are higher than those calculated for the fluorescence signal reduction, demonstrating that the inactivation precedes the conformational change. These findings also suggest that the inactivation is due to the reaction of H$_2$O$_2$ with one of the two cysteines contained in the active site region, producing a reactive intermediate, which successively reacts with the proximal cysteine forming the disulfide bond.

**Fig. 6.** Outline of the three-dimensional structure of IF2 LMW-PTP as determined by x-ray diffraction (31). Two Trp residues and Cys-12 and Cys-17 are reported; the sulfate ion in the active site pocket is also shown. The structure was drawn by WebLab Viewer, version 1.1.

**Fig. 7.** Fluorescence emission spectra of untreated, H$_2$O$_2$-inactivated, and thermally inactivated LMW-PTPs. Spectra were acquired with a Shimadzu model RF-5000 spectrofluorometer. The enzymes were dissolved in 0.1 M Tris-HCl buffer, pH 7.5; H$_2$O$_2$ (final concentration, 0.1 mM) was directly added to the mixtures in the fluorometer cuvettes, and the remaining activity was monitored. The excitation wavelength was 280 nm. The fluorescence emission spectra refer to the active and the partially H$_2$O$_2$-inactivated IF1 LMW-PTP (A), the untreated and the thermally denatured IF1 isoforms (B), the active and the partially H$_2$O$_2$-inactivated IF2 LMW-PTP (C), and the untreated and the thermally denatured IF2 isoform (D).
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TABLE III
Kinetic analysis of inactivation and of conformational change of LMW-PTP

| Inactivation $k_{obs}$  | Conformational change, $k_{obs}$ |
|-------------------------|---------------------------------|
| LMW-PTP-IF1 + 0.1 mM H$_2$O$_2$ | $135 \times 10^{-5} \pm 2 \times 10^{-5}$ s$^{-1}$ |
| LMW-PTP-IF2 + 0.1 mM H$_2$O$_2$ | $199 \times 10^{-5} \pm 4 \times 10^{-5}$ |

*The kinetics of conformational change was determined by recording the fluorescence decrease at $A_{nm}$ during the experimental time. Experiments were performed with 0.1 mM H$_2$O$_2$ (final concentration) in 0.1 M Tris-HCl, pH 7.5.*

**DISCUSSION**

Several important cellular processes are regulated by protein tyrosine phosphorylation. The activation of tyrosine kinase growth factor receptors transiently enhances cellular protein phosphorylation, which is rapidly reverted by the action of potent cellular PTPs, both membrane-bound and soluble enzymes (2). On the other hand, the high number of PTPs codified in the eucaryotic genome suggests that the regulation of cellular protein phosphorylation levels does not depend exclusively on the regulation of protein-tyrosine kinases, but also on the regulation of certain PTPs. LMW-PTPs do not have general sequence homology with other PTPs but share the common active site motif CXXXXXR (C and R are essential catalytic residues) and also have the same catalytic mechanism (2). Whereas many enzymes in the other PTP families possess regulatory or targeting domains, LMW-PTP is composed of a catalytic domain alone. Two LMW-PTP isoforms, produced from a single gene through alternative splicing, are expressed in mammalian cells. Recently, LMW-PTP has been implicated in the regulation of cell signaling started by the activation of PDGF and insulin receptors (3–5). Its activity seems to be regulated by the phosphorylation of Tyr-131 and Tyr-132 by Src family kinases (7, 8).

Some years ago, we proposed that PTPs may be regulated by the transient chemical modification of the essential cysteine contained in the active site. In fact, we observed that nitric oxide reacts in vitro with the PTP essential cysteine, forming an S-nitrosothiol derivative (12). In the case of LMW-PTP, the presence of an additional proximal cysteine within the active site loop determines the formation of a disulfide bond. The reduction of the modified protein with low molecular weight thiols rapidly restores the enzyme activity. Additional experiments performed with macrophages (RAW 264.6 line), stimulated by a bacterial lipopolysaccharide and interferon-γ to produce NO in the cell, demonstrated a strong decrease of both membrane-bound and soluble PTP activities, suggesting that nitric oxide really regulates PTPs in vivo (14).

The results described in this paper reveal an additional mechanism for the regulation of LMW-PTP activity: hydrogen peroxide, produced during cellular oxidative stress conditions and during mitogenic signaling starting from a number of membrane receptors (15), inactivates both LMW-PTP isoenzymes at physiological concentration levels. The inactivation, which is reverted by low molecular weight thiols, is caused by the specific oxidation of both active site loop Cys-12 and Cys-17, which form a disulfide bond. Only these two cysteines out of eight contained in the enzyme (all are in the −SH form (16)) show high reactivity versus hydrogen peroxide. Probably, the presence of two vicinal thiols in the active site region favors the formation of the observed disulfide bridge. A similar event has been previously observed in the mechanism of LMW-PTP inactivation by nitric oxide (12). Very recently, Finkel (10) observed that there is a potential analogy between nitric oxide and other ROS produced in the cell. In both cases, high levels of these reactive compounds are produced by immune effector cells, whereas lower amounts are used by other cell types for signal transduction. We consider it very interesting that both nitric oxide and H$_2$O$_2$ inactivate PTP modifying a unique molecular target, i.e. the essential active site cysteine thiol. Searching the main sequence data banks (GenBank, SWISS-PROT, EMBL, PIR), we have noted that LMW-PTP is the sole PTP containing two cysteine residues within the conserved active site motif (CXXXRX), which is strictly conserved in all members of the large PTP family. In all prokaryotic and eucaryotic organisms, both LMW-PTP Cys-12 and Cys-17 are conserved, indicating that these two vicinal thiols are involved in some peculiar cellular functions or regulations.

With respect to the mechanism of LMW-PTP inactivation by H$_2$O$_2$, we considered two possibilities. Taking into account that H$_2$O$_2$ has moderate reactivity, it could act to generate more reactive species, such as the hydroxyl radical produced in vitro by metal-dependent breakdown of H$_2$O$_2$ (17) (trace metal may be present in the buffer). Alternatively, considering that the catalytic Cys-12 of LMW-PTPs has a remarkably low pK$_a$ (18) and therefore it is in the thiolate form at physiological pH values, we would expect that it reacts with H$_2$O$_2$ by a nucleophilic displacement reaction forming a cysteine sulfenic acid; this mechanism was suggested for the catalytic pathways of a number of enzymes in which a catalytic thiolate ion reacts with hydroperoxides (19–22). Thus, we performed LMW-PTP inactivation experiments by H$_2$O$_2$ both in the absence and in the presence of 2 mM EDTA. We found that the addition of this metal chelator to the reaction mixtures causes 17 and 28% decreases of the second-order rate constants for H$_2$O$_2$ inactivation of IF1 and IF2 LMW-PTP isoforms, respectively. These findings demonstrate that the observed inactivation is mainly due to a direct action of H$_2$O$_2$ on the isoenzymes, although the hydroxyl radical also contributes to inactivation.

Denu and Tanner (23) have recently reported that some PTPs are inactivated by H$_2$O$_2$ and that the inactivation is due to the oxidation of the −SH group of the catalytic cysteine, producing a sulfenic acid. Although our H$_2$O$_2$ inactivation experiments, performed with LMW-PTPs using HPLC-mass spectrometry analyses, do not reveal the formation of any sulfenic acid functions, we do not exclude that this is formed as a transient intermediate in the pathway that leads to the production of the disulfide bond found in H$_2$O$_2$-treated LMW-PTPs.

The involvement of physiological low molecular weight thiols and thioredoxin in the transient modification of the enzyme active site in vivo cannot be ruled out. Thomas et al. (24) proposed that cellular proteins react with metabolically generated oxidative compounds forming reactive sulfhydryl intermediates, such as thiol radicals or sulfenic acids. These modifications may be reverted by the rapid reaction with glutathione, forming S-thiolate derivatives, followed by reductive processes that include direct reduction by glutathione or, more likely, by dithiol redox proteins, such as thioredoxin or glutaredoxin.

A growing body of evidence has indicated that cellular redox status modulates various functions in normal and pathological conditions. Oxidative stress has been implicated in a wide range of disease states and degenerative processes, such as cardiovascular diseases, cancer, rheumatoid arthritis, inflammatory states, and aging (25). However, the exact mechanism of ROS insult to cells is not yet well defined. The transient rise in ROS observed following ligand activation of a number of membrane receptors (15) is actually considered very important for signaling processes. In fact, the transient increase in ROS has been observed in vascular smooth muscle cells stimulated by PDGF (26) and in A431 cells stimulated by epidermal...
growth factor (27). Lee et al. (28) found that PTP 1B is transiently inactivated after stimulation of A431 cells with epidermal growth factor and suggested that this action, which is concurrent with that performed by the epidermal growth factor-receptor tyrosine kinase, contributes to determining the steady-state level of protein phosphorylation necessary for growth stimulation. The activation of transcription factor nuclear factor κB, which is stimulated by a host of ligands, is blocked by antioxidant treatment (29), indicating that ROS are implicated in such signaling processes. Very interesting is the work of Schreck et al. (29, 30); they reported that hydrogen peroxide and lipid hydroperoxides, but not the superoxide anion, activate nuclear factor κB in some cell lines.

We think that our findings on the reversible H$_2$O$_2$ inactivation of LMW-PTP, an enzyme involved in the regulation of some signaling pathways starting from tyrosine kinase receptors, may contribute to understanding the mechanisms of some ROS cellular actions.

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