Specific and Reversible Inactivation of Protein Tyrosine Phosphatases by Hydrogen Peroxide: Evidence for a Sulfenic Acid Intermediate and Implications for Redox Regulation†

John M. Denu* and Kirk G. Tanner

Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, Oregon 97201

Received December 10, 1997; Revised Manuscript Received February 19, 1998

ABSTRACT: Protein tyrosine phosphatases (PTPs) catalyze the hydrolysis of phosphotyrosine from specific signal-transducing proteins. Although regulatory mechanisms for protein kinases have been described, no general mechanism for controlling PTPs has been demonstrated. Numerous reports have shown that cellular redox status plays an important role in tyrosine phosphorylation-dependent signal transduction pathways. This study explores the proposal that PTPs may be regulated by reversible reduction/oxidation involving cellular oxidants such as hydrogen peroxide (H_2O_2). Recent reports indicated that H_2O_2 is transiently generated during growth factor stimulation and that H_2O_2 production is concomitant with relevant tyrosine phosphorylation. By use of recombinant enzymes, the effects of H_2O_2 on three PTPs [PTP1, LAR (leukocyte antigen-related), and VHR (vaccinia H1-related)] and three distinct serine/threonine protein phosphatases (PPs: PP2Cα, calcineurin, and λ phosphatase) were determined. Hydrogen peroxide had no apparent effect on PP activity. In contrast, PTPs were rapidly inactivated ($k_{inact} = 10-20\ M^{-1}\ s^{-1}$) with low micromolar concentrations of H_2O_2 but not with large alkyl hydroperoxides. PTP inactivation was fully reversible with glutathione and other thiols. Because of the slower rate of reduction, modification occurred even in the presence of physiological thiol concentrations. By utilization of a variety of biochemical techniques including chemical modification, pH kinetic studies, and mutagenesis, the catalytic cysteine thiolate of PTPs was determined to be the selective target of oxidation by H_2O_2. By use of the electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), it was shown that a cysteine sulfenic acid intermediate (Cys-SOH) is formed after attack of the catalytic thiolate on H_2O_2. A chemical mechanism for reversible inactivation involving a cysteine sulfenic acid intermediate is proposed.

Within the cell, the steady-state and transiently induced levels of protein phosphorylation on serine/threonine/tyrosine residues are under the strict control of protein kinases and protein phosphatases. These enzymes are in turn, regulated by diverse mechanisms. Many serine/threonine and tyrosine protein kinases are directly controlled by reversible phosphorylation (*1*). The serine/threonine protein phosphatases are generally regulated through their association with other regulatory subunits (*2*−*4*). For protein tyrosine phosphatases (PTPs),[1] a general mechanism for regulation of enzymatic activity has yet to be demonstrated. Unlike many protein kinases, PTPs do not appear to require posttranslational modification for catalytic activity (*5*), since recombinant PTPs expressed in bacteria exhibit high catalytic efficiency. Therefore, it is reasonable to propose that PTPs may be controlled by negative mechanisms. General protein inhibitors or regulatory subunits of PTPs have not been described, suggesting that PTP activity may be controlled by a novel mechanism.

Increasing numbers of reports have indicated that cellular redox status plays an important role in the mechanisms that regulate the function of growth factors and tyrosine phosphorylation-dependent signal transduction pathways (*6*−*11*). Also, intracellular redox-active species have been shown to independently mediate signaling events. Oxidants and thiol-directed agents cause a rapid increase in the level of tyrosine phosphorylation in treated cells (*9*, *12*−*17*). It has been suggested that these effects are the result of inhibition of intracellular PTPs (*17*−*19*); however, direct evidence is lacking. Hydrogen peroxide (H_2O_2) has been shown to stimulate a complete program of mitogenic signal transduction. Hydrogen peroxide-treated cells mimic the phosphorylation cascades that are induced by several growth factors such as epidermal growth factor (EGF) (*13*) and platelet-derived growth factor (PDGF) (*16*). Potent activation of extracellular regulated protein kinase (ERK) is observed immediately after H_2O_2 treatment (*20*). Endogenously produced H_2O_2 was shown to regulate Src family kinases in adherent neutrophils (*21*).

† This work was supported by American Cancer Society Grant RPG-97-175-01-TBE and the Medical Research Foundation of Oregon.

* Corresponding author: Department of Biochemistry and Molecular Biology L224, Oregon Health Sciences University, 3181 SW Sam Jackson Park Rd., Portland, OR 97201-3098. Phone (503) 494-0644; Fax (503) 494-8393; email denuj@ohsu.edu.

[1] Abbreviations: PTPs, protein tyrosine phosphatases; PPs, serine/threonine protein phosphatases; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; ERK, extracellular regulated protein kinase; MAP kinase, mitogen-activated protein kinase; *p*NPP, *p*-nitrophenyl phosphate; β-ME, β-mercaptoethanol; GSH, reduced glutathione; DTT, dithiothreitol; PVDF, poly(vinylidene fluoride); PAGE, polyacrylamide gel electrophoresis; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole.

S0006-2960(97)03035-3 CCC: $15.00
Published on Web 04/02/1998

Recent reports have indicated that the cellular production of H_2O_2 is involved in normal receptor-mediated signal transduction. In vascular smooth muscle cells, PDGF transiently increased the intracellular concentration of H_2O_2 (*16*). When growth factor stimulation of intracellular H_2O_2 production was blocked, tyrosine phosphorylation, MAP kinase activation, DNA synthesis, and chemotaxis were inhibited. Similarly, EGF treatment induced the transient intracellular generation of H_2O_2 in human epidermoid carcinoma cells (*22*). The tyrosine kinase activity of EGF receptor was required for the production of H_2O_2. Given these recent observations, there is evidence that H_2O_2 is generated by receptor-mediated events and thereby may play a critical role in signal transduction.

In this study we explore the idea that PTPs are controlled by redox mechanisms and that PTPs are a viable target of the transient production of intracellular H_2O_2. We propose that reversible oxidation and reduction of a single catalytic residue may be an important regulatory mechanism for downregulating PTPs during mitogenic stimulation. To examine the scope of this potential mechanism of regulation, we investigated three PTPs (PTP1, LAR, and VHR). PTP1B and LAR have been implicated in downregulating the insulin receptor (*23*−*28*). Other evidence suggests that PTP1B might also regulate the EGF receptor (*29*). The functions of VHR are not yet known. Three distinct serine/threonine protein phosphatases were also examined to illustrate that this redox modification was intrinsic to PTPs. The experiments described here demonstrate that PTPs react rapidly with low micromolar concentrations of H_2O_2, specifically oxidizing the essential catalytic cysteine residue to a stabilized sulfenic acid (Cys-SOH) intermediate. No effect of H_2O_2 was observed for serine/threonine protein phosphatases PP2Cα, calcineurin (PP2B), and λ phosphatase. Inactivated PTPs can be fully reactivated with physiological thiols, such as reduced glutathione.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes. All chemicals were of the highest grade commercially available. Hydrogen peroxide (30% ACS reagent) was from Fisher. Cumene hydroperoxide (80%) and *t*-butyl hydroperoxide (70%) were from Sigma. Hydroperoxides were diluted daily from concentrated stocks.

VHR was purified as described in ref *30*. PTP1 was a gift from Dr. Jack Dixon, University of Michigan (*31*). LAR and λ phosphatase were purchased from New England Biolabs. The bacterial expression plasmid for human PP2Cα was a gift from Dr. Patricia Cohen, University of Dundee. PP2Cα phosphatase was purified as outlined in (*32*). Calcineurin was a gift from Dr. Tom Soderling, Vollum Institute, Oregon Health Sciences University. For experiments requiring the absence of reducing agents, DTT was removed from VHR and PTP1 preparations by size exclusion chromatography on a Beckman BioSys 510 system with a TosoHaas TSK-gel G3000SW (7.5 mm × 30 cm) or by extensive dialysis.

Inactivation of Protein Phosphatases by Hydrogen Peroxide. Inactivation of protein phosphatases by H_2O_2 was assayed as the decrease in phosphatase activity against the universal phosphatase substrate *p*-nitrophenyl phosphate (*p*NPP). Phosphatase activity was assayed by monitoring the *p*-nitrophenolate absorbance at 410 nm in a Shimadzu BioSpec-1601 UV−vis spectrophotometer. All assays were performed at pH 7 in a three-component buffer containing 0.1 M acetate, 0.05 M Bis-Tris, and 0.05 M Tris (*33*). VHR (0.49 μM) and PTP1 (0.02 μM) and LAR (0.17 μM) were reacted with 0.9 mM H_2O_2 in the absence of reducing agents. All phosphatases (VHR, LAR, PTP1, PP2Cα, λ phosphatase, and calcineurin) were reacted with 0.9 mM H_2O_2 in the presence of 1 mM DTT. For PP2Cα and λ phosphatase, $MnCl_2$ at 5 mM was added to the buffer. With calcineurin (42 nM), 0.1 mM $CaCl_2$, 6 mM $MgCl_2$, and 470 nM calmodulin were added to activate the enzyme. Also, cumene hydroperoxide and *t*-butyl hydroperoxide at 0.9 mM were reacted with PTP1 and VHR (Table 1). The apparent rate of inactivation was calculated by two separate methods. In a continuous method, inactivation was followed as an exponential decay of activity (Figure 1) and the data were fitted to $\text{Abs} = (\text{Amp})e^{-kt} + B$, where Abs is absorbance at 410 nm, Amp is the change in absorbance, k is the first-order rate constant, t is time, and B is the starting absorption if not 0. Alternatively, an end point assay was utilized as is described below for generating the pH profile of inactivation. To continuously monitor the inactivation and reactivation (Figures 1 and 2), the substrate *p*NPP was present at 0.5 mM during H_2O_2 inactivation and was 20 mM during reactivation. In all subsequent kinetic studies, *p*NPP was omitted during inactivation and reactivation.

Table 1: Kinetic Analysis of H_2O_2 Inactivation of PTPs and PPs[a]

enzyme	peroxide	k_{inact} (M^{-1} s^{-1})
	Protein Tyrosine Phosphatases	
VHR	H_2O_2	17.9 ± 1.3
	t-butyl hydrogen peroxide	no effect[b]
	cumene hydrogen peroxide	no effect[b]
PTP1	H_2O_2	9.1 ± 0.1
	t-butyl hydrogen peroxide	no effect[b]
	cumene hydrogen peroxide	no effect[b]
LAR	H_2O_2	14.0 ± 3.1
	Serine/Threonine Protein Phosphatases	
calcineurin, PP2B	H_2O_2	no effect[b]
λ Phosphatase	H_2O_2	no effect[b]
PP2Cα	H_2O_2	no effect[b]

[a] The k_{inact} constant with VHR was determined from the pH profile (Figure 4) as described in Materials and Methods. For PTP1 and LAR, the k_{inact} values were determined at pH 7. [b] No significant effects on phosphatase activity were observed at 1 mM peroxide.

Dephosphorylation assays with the synthetic peptide DDENleTGpYVATR were performed as described previously (*30*). VHR (0.39 μM) was reacted with 0.9 mM H_2O_2 and assayed for enzymatic activity against the phosphorylated peptide DDENleTGpYVATR (0.56 mM), corresponding to the activation loop of p38 MAP kinase.

Reactivation Kinetics of Hydrogen Peroxide-Inactivated PTPs Using Various Thiols as Reducing Agents. Hydrogen peroxide at 800 μM was added to 0.90 mL of the pH 7 three-component buffer containing VHR (0.16 μM) or PTP1 (0.88 μM). The H_2O_2 inactivation of VHR was allowed to proceed for 10 min at 25 °C prior to depleting H_2O_2 by the addition of catalase (160 units). Reduced thiols were added to final concentrations of 10 mM (β-ME and Cys) for VHR experiments and 7 mM (DTT) and 14 mM (β-ME and GSH) for PTP1 experiments. Phosphatase activity was assayed with *p*NPP at a final concentration of 20 mM. For establishing

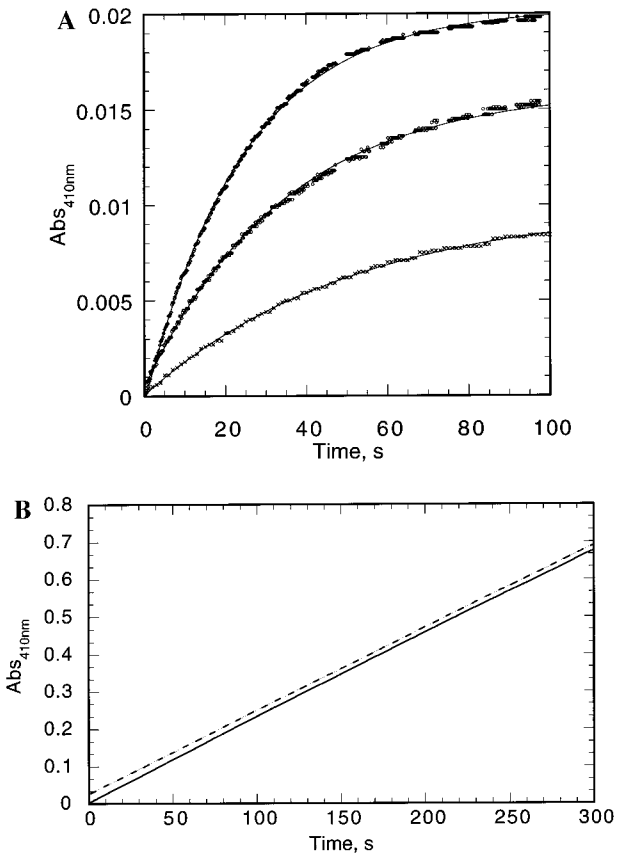


FIGURE 1: Rapid inactivation of PTPs by hydrogen peroxide but not alkyl hydroperoxides. (A) PTP1 (◇, top curve), VHR (○, middle curve), and LAR (×, bottom curve) were inactivated by the addition of 0.9 mM H_2O_2 in the absence of reducing agents. The catalytic activity was followed by monitoring the production of *p*-nitrophenolate ion at 410 nm from the hydrolysis of *p*-nitrophenyl phosphate. Complete inactivation was observed after ~100 s. (B) No inactivation was observed when either *t*-butyl hydroperoxide (solid line) or cumene hydroperoxide (dashed line) was added to the assay at 0.9 mM. Data obtained with VHR are displayed. Conditions: 100 mM acetate, 50 mM Bis-Tris, and 50 mM Tris, pH 7 and 30 °C.

the stability of the inactivated enzyme, VHR was incubated at 25 °C in the inactivated state for periods of time spanning 10 min to 180 min before the addition of 30 mM β-ME. Recoverable activity was determined after 30 min of β-ME treatment. To determine the extent of spontaneous reactivation, phosphatase activity was calculated after 60 min in the absence of thiols (Table 2).

[^{14}C]Carboxymethylation Experiments. Hydrogen peroxide at 1 mM or a pH 6 buffer control was added to the pH 6 three-component buffered solution containing either wild-type VHR (34 μM) or C124S (5.6 μM). The solutions stood at 25 °C for 10 min prior to the addition of [^{14}C]iodoacetic acid (ICN, 13.4 mCi mmol^{-1}) at a final concentration of 2.4 mM. After 30 min the carboxymethylation reactions were quenched by addition of 100 mM DTT. After an additional 5 min, the proteins were unfolded in a solution of SDS for 2 min at 95 °C prior to resolving the protein bands by electrophoresis on a 12% polyacrylamide gel cast in a solution of 0.1% SDS. The polypeptides in the gels were then electrotransferred (*34*) to membranes of PVDF for 45 min at 125 V. The membranes of PVDF were placed on a Bio-Rad CS imaging screen and exposed for 24−72 h. Imaging was performed on a Bio-Rad GS-525 Molecular Imager system and the data were acquired using Bio-Rad Molecular Analyst software.

pH Profile of the Rate of Inactivation by Hydrogen Peroxide. Hydrogen peroxide was added at final concentrations of 45, 150, and 300 μM to samples of VHR (5 μM) in a three-component buffer (final volume of 0.1 mL) that had been preincubated at 30 °C for 3 min. Aliquots (10 μL) were removed at intervals spanning 15 s to 1200 s from the initial addition of H_2O_2 and added to a pH 6 three-component buffer (0.3 mL) containing 20 mM *p*NPP and 57 units of catalase to eliminate the residual H_2O_2. The solutions remained at 30 °C for 5 min prior to the addition of 0.7 mL of 1.0 N NaOH to stop the reaction and the absorbances at 410 nm were read. The log of the fraction of remaining activity was plotted as a function of time for each concentration of H_2O_2. At each pH value, the slope of these decays were plotted as a function of H_2O_2 concentration to yield the apparent second-order rate constant of inactivation. The data were fitted using the equation $\log (v) = \log C/(1 + H/K_a)$, where v is the apparent second-order rate constant, C is the pH-independent rate constant, H is the hydrogen ion concentration, and K_a is the ionization constant for a group that must be unprotonated.

Modification of Hydrogen Peroxide-Treated and Non-treated Samples of VHR with NBD-Cl. Hydrogen peroxide was added to a final concentration of 0.9 mM to samples of wild-type VHR (20 μM) and C124S VHR (60 μM) in 50 mM KCl and 20 mM Bis-Tris, pH 7.0 (final volume of 0.44 mL), that had been preincubated at 25 °C for 3 min. These preparations of VHR were previously treated with DTT (final concentration of 1 mM) for 20 min at 30 °C to fully reduce all cysteine residues. The DTT was then removed by dialysis against four changes of 50 mM KCl and 20 mM Bis-Tris, pH 7.0, over 80 min. After 15 min, 100 units of catalase was added to eliminate the residual H_2O_2. Catalase was also added to the non-H_2O_2-treated samples. After 3 min of catalase treatment, NBD-Cl (in dimethyl sulfoxide) was added to a final concentration of 0.8 mM. The modification reactions were then allowed to proceed at 25 °C for 1 h. The samples were then dialyzed extensively against four changes of 20 mM sodium phosphate (pH 7.2) with 150 mM NaCl. The absorbance spectra from 750 to 250 nm were recorded in a Shimadzu BioSpec-1601 UV−vis spectrophotometer.

RESULTS

The goals of this investigation were to provide biochemical evidence for the proposal that PTPs may be reversibly regulated by cellular oxidants, to demonstrate that the catalytic cysteine of PTPs is the specific target of reversible H_2O_2 oxidation and to elucidate the chemical mechanism for this reaction. The well-characterized PTPs VHR, LAR, and PTP1 were employed as prototypes. VHR represents a member of the dual-specificity PTPs, proteins capable of hydrolyzing phosphomonoesters of tyrosine and serine/threonine (*35*). PTP1 represents an intracellular and tyrosine-specific PTP, while LAR represents the receptorlike PTPs. The X-ray structures of human PTP1B and VHR have been solved (*36*, *37*). The enzyme PTP1 used in this study is the rat homologue of PTP1B. Previous kinetic, biochemical, and structural analyses of these enzymes have revealed a common

reaction mechanism that involves the formation of a transient phosphoenzyme intermediate (*5*). The phosphate group of substrate is transferred to the nucleophilic active-site cysteine residue. Serine replacement of this cysteine yields a enzyme devoid of measurable activity. The pK_a value of the cysteine is 5.5 (*30*, *32*), greater than 3 pH units lower than a normal cysteine residue. At physiological pH values the catalytic cysteine is a thiolate anion.

VHR, LAR, and PTP1 were reacted with 0.9 mM H_2O_2 in the absence of reducing agents (Figure 1A). There was a rapid loss of PTP activity and complete inactivation was observed after ~100 s (Figure 1A). Linear kinetics were observed in the absence of H_2O_2 over the first 10 min. The apparent rates of inactivation under these conditions were very similar at 0.041 ± 0.001 s^{-1} for PTP1, 0.029 ± 0.001 s^{-1} for VHR, and 0.021 ± 0.001 s^{-1} for LAR. To demonstrate whether the effects of H_2O_2 resulted from nonspecific oxidation, other hydroperoxides were examined under identical conditions. Large alkyl hydroperoxides (*t*-butyl hydroperoxide and cumene hydroperoxide) at 0.9 mM had no apparent effect on catalytic activity (Figure 1B), suggesting that inactivation of PTPs by H_2O_2 is relatively specific. If the reaction with H_2O_2 involves the modification of the active-site cysteine, the bulkier alkyl hydroperoxides would be predicted to be largely excluded from the active-site cleft (*36*, *38*), consistent with their lack of reactivity with PTPs.

Loss of activity against the synthetic phosphopeptide DDENleTGpYVATR, corresponding to the activation segment on p38 MAP kinase (*1*), was also observed with H_2O_2-inactivated VHR. After 1 min 80% of original activity remained, and after 10 min less than 3% remained. The time course of inactivation was in agreement with that assayed with *p*NPP as substrate.

We reasoned that if H_2O_2 inactivation was restricted to the catalytic cysteine of PTPs, then the serine/threonine protein phosphatases (PPs) should be largely insensitive to treatment with H_2O_2. The PPs are metal-dependent protein phosphatases that do not appear to form a phosphoenzyme intermediate during catalysis. It is proposed that a metal-bound water molecule, acting as nucleophile, directly attacks the phosphate of substrate (*39*). To examine the H_2O_2 sensitivity of PPs, we attempted to inactivate members (human PP2Cα, λ phosphatase, and calcineurin) from the two known gene families of PPs (Table 1). PP2Cα is a member of the PP2C family of PPs, while calcineurin (PP2B) and λ phosphatase are members of the gene family that also includes PP1 and PP2A. During 10 min reactions, PP2Cα, λ phosphatase, and calcineurin exhibited no significant effects from H_2O_2 treatment under the identical conditions that completely inactivated the PTPs (Table 1). These results indicate that PPs are relatively immune to H_2O_2 oxidation. PTPs, however, harbor an intrinsic reactivity with H_2O_2 and are rapidly oxidized at low micromolar concentrations.

Next, we investigated whether H_2O_2 inactivation was reversible by using thiols to reactivate H_2O_2-treated PTP1, LAR, and VHR. The PTPs were first inactivated by H_2O_2 and the excess H_2O_2 was removed by the addition of catalase. Various thiols were then added and the reactivation of enzymatic activity was monitored using *p*NPP as substrate (Figure 2). Dithiothreitol (DTT), β-mercaptoethanol (β-ME), reduced glutathione (GSH), and cysteine (at 10−14 mM thiol equivalents) were all capable of reactivating the PTPs (Figure 2). Reactivation with DTT was significantly faster than with other thiol reagents at equivalent thiol concentrations. Faster reactivation with DTT would be expected if an oxidized cysteine residue requires 2 thiol equiv to recover full activity. Approximately 85% of the original enzymatic activity of both VHR and PTP1 was reactivated with thiol reducing agents. LAR was also reactivated but only ~10% of the original activity was recovered due to the extreme instability of LAR, even in the absence of H_2O_2.

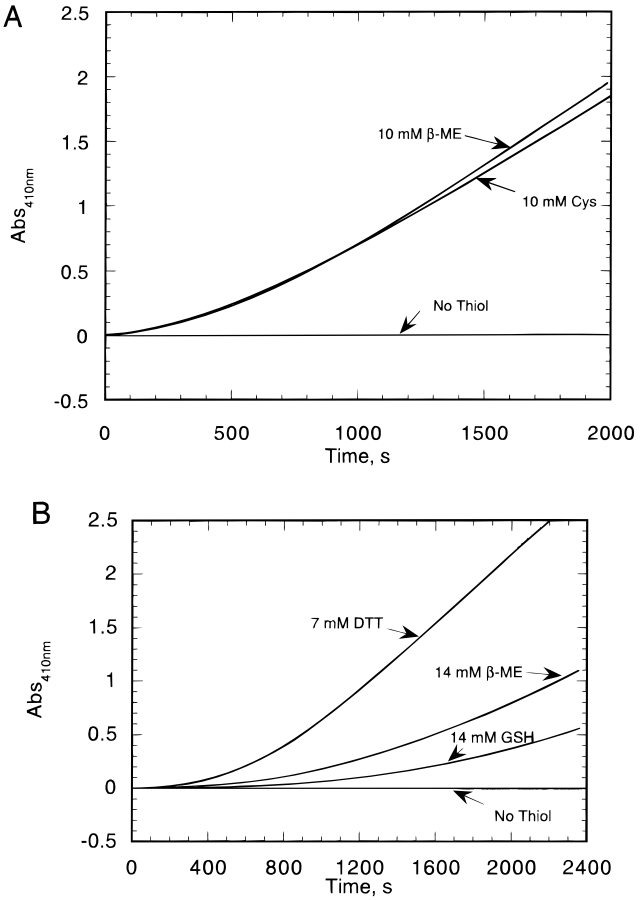


FIGURE 2: Reactivation kinetics of hydrogen-peroxide inactivated PTPs using various thiols as reducing agents. VHR (A) and PTP1 (B) were first inactivated with H_2O_2 (0.8 mM) and the excess H_2O_2 was removed by catalase. The PTPs were then reactivated by reducing agents at 10 mM (A) and 14 mM (B) thiol equivalents. Concentrations of VHR and PTP1 were 0.16 and 0.88 μM, respectively. The activity was followed as described in Figure 1 and in Experimental Procedures.

Inactivation could not be prevented in the presence of reducing agents. The addition of 1 mM DTT slowed the inactivation by ~3−10-fold but did not prevent inactivation with 0.9 mM H_2O_2. These data suggested that a slight molar excess of DTT over H_2O_2 did not provide ultimate protection from PTP oxidation, suggesting that H_2O_2 reacts more rapidly with enzyme than with DTT. Rapid reaction with the PTP catalytic thiolate is consistent with previous studies on the increased H_2O_2 reactivity of thiolates versus protonated thiols. Under the conditions of the reaction (pH 7), DTT (pK_a > 8) would be protonated and therefore less reactive than the active-site thiolate anion.

In an effort to characterize the inactivated enzyme, we determined the stability of H_2O_2-treated VHR. That is, we determined the effect of time on the ability of H_2O_2-inactivated enzyme to reactivate with thiols. VHR was the

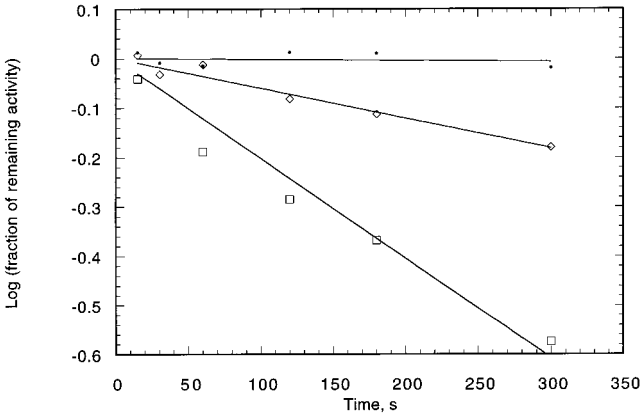


FIGURE 3: Competitive inhibitor phosphate protects PTPs from inactivation by hydrogen peroxide. Addition of the competitive inhibitor phosphate (10 mM, $K_i = 1$ mM) during H_2O_2 (0.1 mM) inactivation of VHR protected the enzyme against modification. The ● symbols represent no H_2O_2 or phosphate. The ◇ symbols represent the addition of 10 mM phosphate with 0.1 mM H_2O_2. The □ symbols represent 0.1 mM H_2O_2 alone. Phosphate also protects PTP1 from H_2O_2 inactivation (see Results).

best choice since the native enzyme was substantially more stable than the other PTPs at 25−30 °C. No decrease in activity was observed for untreated VHR over the course of the experiment. VHR was reacted with 0.8 mM H_2O_2 for 10 min. At various times between 10 and 180 min, the enzyme was reactivated with β-ME and the fraction of remaining activity was determined. Amazingly, even after 120 min, greater than 80% of the original activity was recovered and no significant drop in activity was observed with time. In the absence of reducing agents, no spontaneous reactivation occurred. These results indicate that the inactive enzyme is remarkably stable and does not undergo further oxidation, even in the absence of reducing agents and under aerobic conditions.

To determine if the active site of PTPs is the target of H_2O_2-dependent inactivation, the competitive inhibitor phosphate was included to provide protection from inactivation. Phosphate effectively protected VHR from inactivation (Figure 3). The concentration of phosphate was 10 mM, 10-fold higher than the inhibition constant K_i for phosphate (*33*). Phosphate at an 8-fold excess over the K_i value was also effective at protecting PTP1 from inactivation. Moreover, the presence of substrate slows the apparent rates of both inactivation and reactivation. In a fashion analogous to phosphate, *p*NPP can compete at the active site with H_2O_2 during inactivation and with thiol compounds during reactivation. These data indicate that the active site is the target of H_2O_2. Taken together with the observation that inactivation is fully reversible with the addition of thiol reducing agents, these data suggested that the specific target of H_2O_2 may be the catalytic cysteine.

To demonstrate that the active-site cysteine thiolate anion is the specific target of H_2O_2 inactivation, the pH dependence of the rate of inactivation was determined and the resulting pK_a value was compared to that obtained with the thiol-modifying reagent iodoacetate. It was previously shown that iodoacetate will selectively carboxymethylate the catalytic cysteine-124 of VHR (*40*). No other cysteine residues were modified. The specificity of this modification was utilized to determine the pK_a value of 5.6 ± 0.1 for cysteine-124

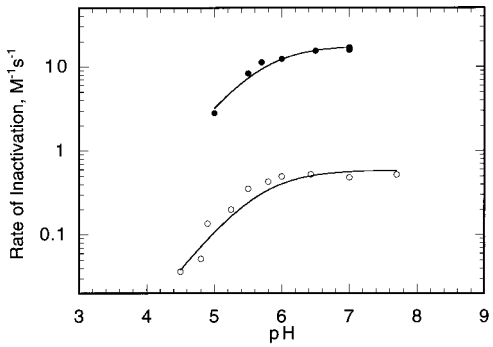


FIGURE 4: pH Profile of the rate of inactivation by hydrogen peroxide. The apparent second-order rate constant for H_2O_2 inactivation of VHR (●) was determined as described in Experimental Procedures. For comparison, the previously determined pH profile for iodoacetate inactivation is also displayed (○) (*28*). Both curves yielded a pK_a value of 5.6 ± 0.1 for an amino acid that must be unprotonated for inactivation. Cysteine-124 of VHR was shown to be the selective target of iodoacetate (*39*).

(*33*). Similarly, the apparent second-order rate constant for H_2O_2 inactivation of VHR was measured as a function of pH (Figure 4). Hydrogen peroxide concentrations as low as 45 μM were effective at inactivating the enzyme. The pH profile for the rate of inactivation by H_2O_2 indicated that an ionizable group on the enzyme must be unprotonated for reactivity (Figure 4). The data yielded a pK_a value of 5.6 ± 0.1 (Figure 4 and Table 1), which is identical to the pK_a value of 5.6 ± 0.1 generated for the specific iodoacetate reaction with cysteine-124 (*33*). The excellent agreement in pK_a values supports the idea that H_2O_2 selectively targets the active-site cysteine residue of PTPs and that the thiolate anion is the reactive species.

To provide additional evidence that the catalytic cysteine is the target of H_2O_2, [^{14}C]iodoacetate was used to demonstrate that H_2O_2-inactivated VHR cannot be modified at the active-site cysteine. If Cys-124 is oxidized by H_2O_2, iodoacetate will not carboxymethylate the enzyme. VHR was inactivated by H_2O_2 and subsequently reacted with [^{14}C]-iodoacetate. VHR was then subjected to SDS−PAGE, electrotransferred to membranes of PVDF, and imaged (Figure 5). Untreated VHR efficiently incorporated [^{14}C]-iodoacetate (Figure 5, lane 3), whereas H_2O_2-inactivated VHR (Figure 5, lane 4) incorporated almost negligible amounts (20-fold less than VHR) of [^{14}C]iodoacetate. In control experiments, no [^{14}C]iodoacetate incorporation was observed with the mutant C124S, with or without H_2O_2-treatment (Figure 5, lanes 1 and 2). Loss of the ability of H_2O_2-inactivated VHR to incorporate [^{14}C]iodoacetate provides strong evidence that oxidation of the catalytic cysteine is the direct cause of enzymatic inactivation.

Our next goal was to identify the chemical nature of the oxidized cysteine intermediate. Since PTP oxidation by H_2O_2 was fully reversible, the intermediate was unlikely to be a sulfinic (−SO_2H) or sulfonic acid (SO_3H), both of which cannot be reduced with thiol agents (*41*). Two other logical possibilities existed. The enzyme could form an intramolecular disulfide (−S−S−) or a stable cysteine sulfenic acid (−SOH), both of which can be reduced back to cysteine. An intermolecular disulfide was ruled out from size-exclusion chromatography and nonreducing SDS−PAGE experiments. Detection of sulfenic acids has been problematic due to their extreme instability. When formed, sulfenic acids (−SOH)

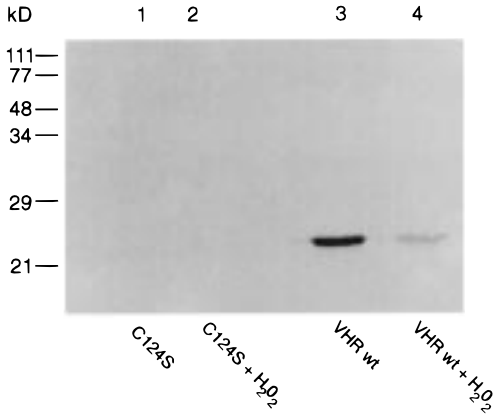
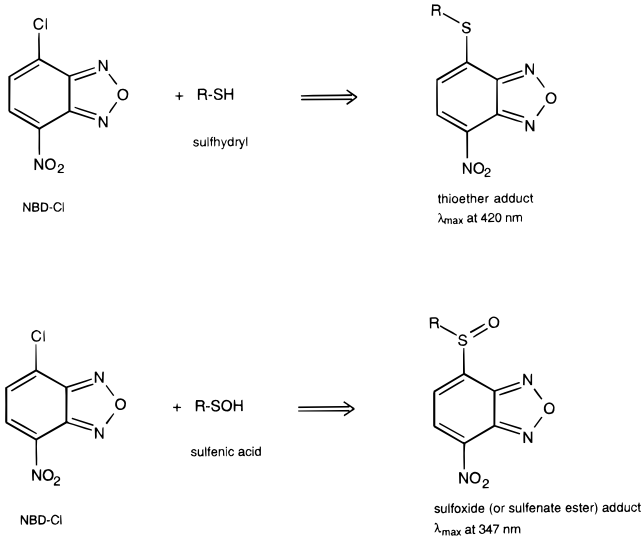


FIGURE 5: Active-site cysteine is the target of hydrogen peroxide-dependent inactivation. As deterimined with the specific active-site cysteine modifying agent iodoacetate ([^{14}C]-labeled), H_2O_2-inactivated VHR lost the ability to react and carboxymethylate Cys-124. VHR (34 μM) and the C124S mutant (5.6 μM) were reacted with 1 mM H_2O_2. Iodoacetate was added at a final concentration of 2.4 mM. Extent of carboxymethylation was measured by [^{14}C]-incorporation into VHR using SDS−PAGE, electrotransfer to membranes of PVDF, and image analysis. Image analysis and quantitation of [^{14}C] counts was performed using the Bio-Rad GS-525 Molecular Imager system (CS imaging screen) and Bio-Rad Molecular Analyst software.

Scheme 1



can readily react with nucleophiles or other free sulfhydryls, making isolation difficult. However, Ellis and Poole have recently described the use of the electrophilic reagent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) to detect and identify cysteine sulfenic acid intermediates at the active sites of a fully active mutant of alkyl hydroperoxide reductase (AhpC) and native NADH peroxidase (*42*). AhpC and NADH peroxidase utilize a cysteine thiolate nucleophile to catalyze the reduction of peroxide substrates with the concomitant formation of a cysteine sulfenic acid. NDB-Cl was shown to react with both the reduced cysteine thiolate and the cysteine sulfenic acid to form two spectrally distinct species, Scheme 1 (*42*). The Cys-S−NBD species absorbs maximally at 420 nm while the Cys-S(O)−NBD species absorbs maximally at 347 nm, allowing clear identification of the two adducts.

Here we have employed this new method to trap a cysteine sulfenic acid intermediate generated from the reaction of

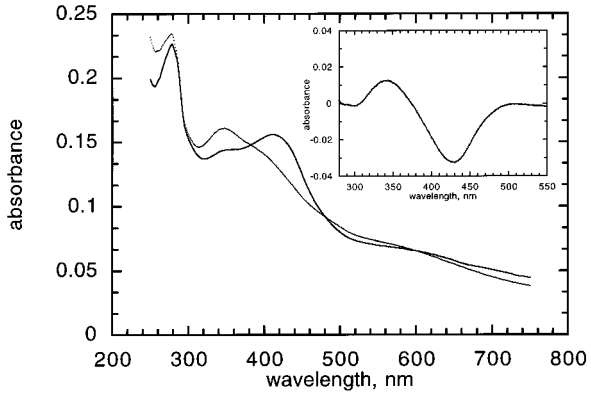


FIGURE 6: Spectroscopic analysis of NBD-modified VHR. The Cys−S−NBD adduct was generated by reacting 20 μM VHR with 0.8 mM NDB-Cl for 1 h, followed by extensive dialysis to remove unreacted NBD-Cl (solid line). The Cys−S(O)−NBD adduct was generated by first reacting VHR with 0.9 mM H_2O_2, then adding 0.8 mM NDB-Cl for 1 h, and subsequent dialysis (dashed line). Catalase was added to each sample prior to the addition of NBD-Cl. (Inset) Difference spectra between the Cys−S−NBD and Cys-S(O)-NBD species. The λ_{max} values for Cys-S(O)−NBD and Cys−S−NBD are 347 and 420 nm, respectively.

H_2O_2 with PTP. VHR and H_2O_2-inactivated VHR were reacted with NBD-Cl for 1 h and excess NBD-Cl was removed by extensive dialysis. The spectra (250−750 nm) of the resulting NBD adducts were determined and are displayed in Figure 6. The characteristic Cys-S−NBD peak centered at 420 nm was observed for the reaction between native VHR and NBD-Cl (solid line, Figure 6). Incorporation of the NBD label into the native enzyme corresponded with the subsequent loss of PTP activity, suggesting that the active-site Cys-124 was being modified. When NBD-Cl was reacted with H_2O_2-inactivated VHR, the spectra revealed a substantial loss of the Cys-S−NBD peak at 420 nm with the concomitant appearance of a species with an absorbance maximum at 347 nm (dashed line, Figure 6). The 347 nm species is identical to that observed for the Cys-S(O)−NBD adduct of AhpC and NADH peroxidase (*42*), providing strong evidence that VHR forms a stable sulfenic acid upon reaction with H_2O_2. There is a slight shoulder at 347 nm in the spectrum of native VHR−NBD adduct, indicating that a small amount of sulfenic acid is present even in the sample not treated with peroxide. This is not surprising considering that these experiments were performed under aerobic conditions and exogenous thiols are removed just prior to NBD-Cl modification. The two adducts exhibited nearly identical absorbance values. Ellis and Poole (*42*) also observed similar absorbance values for the Cys-S(O)−NBD and Cys-S−NBD conjugates of AhpC. The extinction coefficient of the Cys-S(O)−NBD adduct was estimated to be identical to that of the Cys-S−NBD adduct (*42*). Using the extinction coefficients at 280 nm of 11 500 M^{-1} cm^{-1} for VHR (*30*) and 13 400 M^{-1} cm^{-1} for the NBD protein adduct at either 347 or 420 nm (*42*), we estimate that approximately 0.5 mol of NBD is incorporated. We observed nearly equivalent NBD labeling between the native and H_2O_2-treated enzymes, consistent with a single highly reactive cysteine residue that is converted to a sulfenic acid upon H_2O_2 treatment. However, we were aware that VHR contains four cysteine residues. In the case of AhpC and NADH peroxidase (*42*), the enzymes harbored only one cysteine residue, simplifying the potential complications from the presence of more than

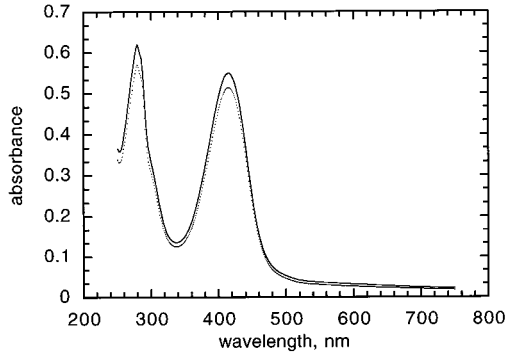


FIGURE 7: Spectroscopic analysis of NBD-modified C124S VHR mutant. The C124S VHR mutant (60 μM) was reacted with 0.8 mM NBD-Cl either after 0.9 mM H_2O_2 treatment (dashed line) or without any H_2O_2 treatment (solid line). Other conditions are identical to those described for wild-type VHR in Figure 6 and in Materials and Methods.

one cysteine residue in the protein. The possibility that a cysteine residue other than the catalytic Cys-124 was forming a Cys-S(O)−NBD adduct in VHR was excluded, as discussed below.

The Cys-S(O)−NBD and Cys-S−NBD adducts of VHR displayed no phosphatase activity. But both conjugates could be reactivated by using β-ME to displace NBD in a reversal of the nucleophilic aromatic substitution reaction. Removal of covalently bound NBD correlated with the reappearance of PTP activity. These data support the idea that NBD-Cl is selectively modifying the catalytic cysteine, Cys-124.

It has been shown that the C124S mutant of VHR is devoid of PTPase activity (*40*). Cys-124 is the catalytic nucleophile, forming a phosphoenzyme intermediate during catalysis (*43*). We used the C124S mutant of VHR to demonstrate that the Cys-S(O)−NBD species absorbing at 347 nm in the wild-type enzyme−NBD adduct (Figure 6) resulted from the direct reaction of Cys-124 and H_2O_2. Similar to the NBD-Cl trapping experiments performed with native VHR, the C124S mutant was treated with and without H_2O_2 and then subjected to NBD-Cl modification. The resulting spectral species were analyzed (Figure 7). As is evident from Figure 7, the spectra were almost superimposable. The solid line represents the C124S mutant while the dashed line represents the H_2O_2-treated C124S mutant. There is negligible difference between the two spectra and clearly no indication of a species absorbing at 347 nm. These data indicate that the C124S mutant lacks the reactive thiolate anion and are consistent with Cys-124 being the residue that forms a sulfenic acid upon reaction with H_2O_2.

DISCUSSION

There is mounting evidence that protein phosphorylation may be controlled by cellular redox mechanisms. Oxidative stress is known to increase specific tyrosine phosphorylation (*7*, *9*, *14*, *44*, *45*). It has been proposed that tyrosine kinases and PTPs may be directly controlled by the redox status of the cell (*6*, *45*−*47*). Several reports have suggested that PTPs are inactivated in vivo by the addition of various membrane-permeant oxidants, leading to enhanced tyrosine phosphorylation. In vitro studies have confirmed the susceptibility of PTPs to oxidation (*48*, *49*). However, in these reports the catalytic cysteine was irreversibly oxidized to $-SO_2H$ and $-SO_3H$ by these strong nonphysiological oxidants. If PTPs are controlled by cellular redox mechanisms, it is highly unlikely that PTPs will be *irreversibly* inactivated during downregulation. More probable, the redox mechanism should allow for reversibility without the need for additional protein synthesis, akin to phosphorylation and glycosylation. Intracellular oxidants such as H_2O_2 have received greater attention as potential mediators and perhaps as second messengers of redox signaling. Given the recent observations that H_2O_2 is generated and may be required in normal growth factor-stimulated signal transduction, we sought to provide chemical and kinetic evidence for the idea that PTPs are a logical and viable target of intracellular H_2O_2, to elucidate the chemical basis for inactivation and to provide initial support for our general proposal that PTPs are regulated in the cell by redox mechanisms. Therefore, we have presented a detailed investigation into the mechanism of reversible inactivation of PTPs by H_2O_2.

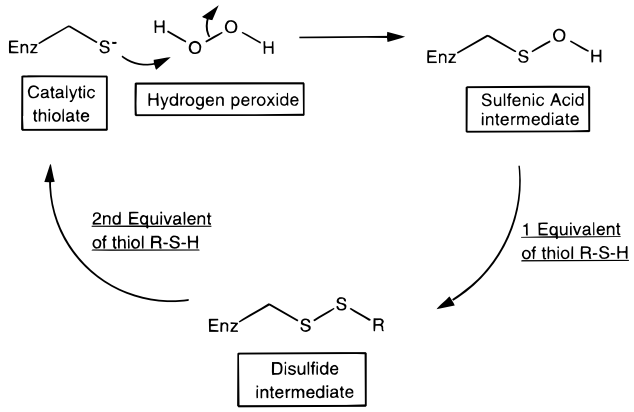
The chemical and kinetic data presented for three different PTPs (PTP1, LAR, and VHR) are entirely consistent with the idea of redox control as a plausible mechanism of regulation and provide the first direct evidence for a cysteine sulfenic acid intermediate. We demonstrated that PTPs rapidly react (10−20 M^{-1} s^{-1}) with H_2O_2, resulting in the complete inactivation of phosphatase activity. Concentrations of H_2O_2 as low as 45 μM were effective at completely inactivating the enzymes. Inactivation of PTPs is fully reversible with thiols, GSH, DTT, β-ME, and cysteine. Because reactivation with thiols is a much slower process than the reaction between H_2O_2 and enzyme, H_2O_2 reacts with PTPs in the presence of excess reducing agents, supporting the notion that PTPs are a target of the transient generation of intracellular H_2O_2, where the reduced glutathione levels are between 1 and 10 mM. We estimate the rate of reactivation by thiols to be 10−100-fold slower than the rate of inactivation (Figures 1 and 2). Additionally, these results suggest that the overall redox state of the cell will dictate the steady-state levels of active enzyme. Also, our chemical and kinetic data are consistent with previous cellular studies. The rapid kinetics of inactivation and the slower kinetics of reactivation are in excellent agreement with in vivo studies on the rapid rise and slower decay of phosphotyrosine levels after growth factor stimulation or after transient H_2O_2-dependent stimulation (*13*, *16*, *20*). Our results are also consistent with the fast decrease and the subsequent slow recovery of general PTP activity following H_2O_2 treatment of fibroblast cells (*19*). Recovery of PTP activity was not affected by cycloheximide, a protein synthesis inhibitor, and an inhibitor of glutathione synthesis retarded the recovery of PTP activity. Transient levels of H_2O_2 induced by growth factor stimulation would be an ideal mechanism to rapidly inactivate PTPs that are present and regulate these pathways. Slower reactivation of PTPs by cellular reduced thiols would ensure that the signal is maintained long enough to elicit the proper response. For reactivation, the most likely intracellular reduced thiol would be glutathione (GSH) since its concentration in the cell ranges from 1 to 10 mM (*50*) and it is known to be the major determinant of cellular redox potential. As we have shown, GSH is an effective agent for reactivation of inactivated PTPs.

Using a variety of methods, we have demonstrated that the essential active-site cysteine of PTPs is the specific target

of H_2O_2-dependent inactivation and that the cysteine thiolate is the reactive species. The initial reaction of the cysteine thiolate with H_2O_2 yields the sulfenic acid of cysteine, which can be reduced back to the active thiolate species. Oxidation to the sulfenic acid renders the enzyme inactive against phosphorylated substrates. During the normal phosphatase reaction, the thiolate nucleophile attacks the electrophilic phosphorus atom of substrate, transferring the phosphate group to enzyme and forming an enzyme thiol-phosphate intermediate (*43*). Because of the abnormally low pK_a value of 5.5 for this cysteine, the thiolate is the predominant form under physiological pH values. Being intrinsically strong nucleophiles, thiolates are susceptible to oxidation by H_2O_2. There is precedent for the increased reactivity of H_2O_2 with protein thiolates. Specific reactions of catalytic protein thiolate ions with hydroperoxides have been demonstrated for NADH peroxidase (*51*), alkyl hydroperoxide reductase (*52*), monoalkylated glutathione reductase (*53*), and others (*54*, *55*). Interestingly, the peroxidases and the reductases catalyze the physiological reduction of hydroperoxide, utilizing an active-site cysteine thiolate. As part of the catalytic pathway, it has been proposed that nucleophilic attack on hydroperoxide results in the formation of a cysteine sulfenic acid (R-SOH) intermediate. In NADH peroxidase, the cysteine sulfenic acid is stabilized within the active-site pocket, as was revealed when the X-ray structure of this intermediate was solved (*51*). With alkyl hydroperoxide reductase, a sulfenic acid is proposed to form transiently along the catalytic pathway (*52*). Recently Ellis and Poole (*42*) have developed a method for trapping the cysteine sulfenic acid of NADH peroxidase and AhpC. This method takes advantage of the different spectral properties of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) after the formation of a covalent adduct to either the cysteine or the sulfenic acid of cysteine. In the present study, we have utilized this method to provide strong evidence for the formation of a stable sulfenic acid intermediate after the inactivation of PTP by H_2O_2. Because sulfenic acids are highly reactive and readily undergo further oxidation, their detection in proteins has been elusive. We provide the first direct evidence that PTPs initially form a sulfenic acid intermediate upon reaction with H_2O_2. This is highly significant since cysteine sulfenic acids (−SOH) are fully reducible back to cysteine, unlike sulfinic acid (−SO_2H) and sulfonic acid (−SO_3H), which have been observed in PTPs (*48*, *49*).

We propose a plausible chemical mechanism (depicted in Scheme 2) for the reversible inactivation of PTPs by H_2O_2. After rapid nucleophilic attack of the thiolate anion on H_2O_2, a cysteine sulfenic acid intermediate is formed and is stabilized in the active-site cleft (Scheme 2). In the presence of reducing thiols, one thiol R-SH equivalent reacts with the sulfenic acid to form a mixed disulfide (Scheme 2). A second thiol R-SH would react with the enzyme mixed disulfide to regenerate active reduced enzyme. We estimate that reactivation in excess thiol reducing compounds is 10−100-fold slower than H_2O_2 inactivation. The rate-limiting step during reactivation is not known. Sulfenic acids are thought to readily react with free thiols, suggesting that perhaps reaction with the second thiol R-SH is the slow kinetic step.

Scheme 2



The observation that H_2O_2-treated VHR can be reactivated with thiols after 3 h without significant loss in activity is consistent with a stabilized sulfenic acid intermediate. Because H_2O_2 oxidation of PTPs is fully reversible over long periods of time, oxidation of cysteine beyond sulfenic acid is unlikely. Sulfinic acid (−SO_2H) and sulfonic acid (−SO_3H) cannot be reduced back to cysteine, whereas sulfenic acids are completely reducible (*41*). It is quite amazing that VHR does not undergo further oxidation of Cys-124 in the presence of 0.27 mM dissolved atmospheric oxygen and excess H_2O_2. This is in marked contrast to AhpC, where the sulfenic acid was trapped using stoichiometric hydrogen peroxide and performing the reaction under anaerobic conditions (*42*). Irreversible oxidation of the catalytic cysteine is observed under such conditions (*42*). With NADH peroxidase, exposure to excess H_2O_2 results in the oxidation of the catalytic sulfenic acid (*56*). In VHR, the cysteine sulfenic acid may be protected in the active-site pocket, sequestered from bulk solution such that further oxidation is not permitted.

Our results clearly indicate that a cysteine sulfenic acid is formed during the reaction of PTP and H_2O_2. During oxidative stress or perhaps growth factor-mediated signaling where PTPs will be exposed to oxidants such as H_2O_2, PTPs will be inactivated by the initial formation of the sulfenic acid at the catalytic cysteine. Reversing this process will depend on the redox status of the cell, the availability of reduced thiols, and the reaction kinetics. The sulfenic acid will then react with reduced glutathione to regenerate the active enzyme (Scheme 2). If the cell requires faster reactivation, it is tempting to suggest that this process may be facilitated by a thioredoxin-type mechanism.

We examined the possibility of a redox mechanism where the cysteine sulfenic acid could react with a different cysteine residue within the same protein to form an intracellular disulfide. In the mechanistically related low molecular weight phosphatase, the catalytic cysteine forms a disulfide bond with a proximal active-site cysteine after oxidation by nitric oxide (*57*). Within this family of phosphatases, both cysteine residues are conserved. Similarly, it was observed that the SH2 containing PTP SHP-1 forms an intramolecular disulfide after reaction with aromatic disulfides (*58*). The authors suggested that the catalytic cysteine was involved since the reaction lead to inactivation, although the residues were not identified (*58*). Several lines of evidence argue against a general mechanism of intramolecular disulfide

formation among PTPs. First, in the entire family of PTPs only the catalytic cysteine is absolutely conserved. Second, the NBD-Cl trapping experiments clearly demonstrated that VHR forms a stable sulfenic acid after H_2O_2 inactivation. Disulfides cannot react with NBD-Cl (*42*). In addition, the catalytic cysteine residues of VHR (*37*) and PTP1B (*36*) are buried at the base of the active-site pocket and inaccessible to other cysteine residues within the same molecule. The X-ray structure of VHR (*37*) and PTP1B (*36*) revealed that there are no other cysteine residues within 9 Å of the catalytic cysteine and that these cysteine residues are themselves buried, making intramolecular disulfide formation highly improbable. Although a general mechanism of intramolecular disulfide formation does not appear to be operating, we would propose that after initial formation of the sulfenic acid, a proximal cysteine could readily react with sulfenic acid to form a disulfide, consistent with our proposed mechanism of redox regulation (Scheme 2). PTPs that might have the potential to form an intracellular disulfide could be predicted from molecular modeling studies and the previously solved X-ray structures.

As proposed in Scheme 2, our results for H_2O_2 inactivation of PTPs are consistent with the formation of a cysteine sulfenic acid, Cys-SOH. Cysteine sulfenic acids have been suggested to function in the redox regulation of the DNA-binding activities of transcription factors Fos, Jun, OxyR, and bovine papillomavirus type 1 E2 protein (*41*, *59*), lending credence to our proposal of redox regulation of PTPs as a viable mechanism. The suggestion that H_2O_2 may act as a second messenger (*9*, *16*) and recent reports that have indicated that the production of H_2O_2 is involved in normal receptor-mediated signal transduction (*13*, *16*, *22*), have led to the logical concept that H_2O_2 and other cellular oxidants are physiological redox regulators of PTPs.

Recent evidence has suggested that serine/threonine protein phosphatases may also be regulated by redox mechanisms (*60*). Wang et al. (*60*) demonstrated that superoxide dismutase prevents the inactivation of calcineurin in vitro and in vivo. Calcineurin is the target of many immunosuppressive drugs and has a critical role in T-cell activation. Superoxide was shown to inactivate calcineurin, probably by oxidizing the catalytic Fe−Zn active center. The authors concluded that the redox state of iron and the cellular redox potential modulate the activity of calcineurin.

ACKNOWLEDGMENT

We thank Dr. Leslie B. Poole for sharing her manuscript on the use of NBD-Cl to trap protein sulfenic acids prior to final acceptance.

REFERENCES

1. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell 85*, 149−158.
2. Shenolikar, S. (1994) *Annu. Rev. Cell Biol. 10*, 55−86.
3. Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T., Cohen, P., and Barford, D. (1997) *EMBO J. 16*, 1876−1887.
4. Cohen, P. (1994) *Bioessays 16*, 583−588.
5. Denu, J. M., Stuckey, J. A., Saper, M. A., and Dixon, J. E. (1996) *Cell 87*, 361−364.
6. Monteiro, H. P., and Stern, A. (1996) *Free Radicals Biol. Med. 21*, 323−333.
7. Flohe, L., Brigelius-Flohe, R., Saliou, C., Traber, M. G., and Packer, L. (1997) *Free Radicals Biol. Med. 22*, 1115−1126.
8. Leclerc, P., de Lamirande, E., and Gagnon, C. (1997) *Free Radicals Biol. Med. 22*, 643−656.
9. Suzuki, Y. J., Forman, H. J., and Sevanian, A. (1997) *Free Radicals Biol. Med. 22*, 269−285.
10. Muller, J. M., Cahill, M. A., Rupec, R. A., Baeuerle, P. A., and Nordheim, A. (1997) *Eur. J. Biochem. 244*, 45−52.
11. Watson, R. W., Rotstein, O. D., Nathens, A. B., Dackiw, A. P., and Marshall, J. C. (1996) *Surgery 120*, 150−158.
12. Natarajan, V., Vepa, S., Verma, R. S., and Scribner, W. M. (1996) *Am. J. Physiol. 271*, 400−408.
13. Rao, G. N. (1996) *Oncogene 13*, 713−719.
14. Suzuki, Y., Ohsugi, K., and Ono, Y. (1996) *Immunology 87*, 396−401.
15. Whisler, R. L., Goyette, M. A., Grants, I. S., and Newhouse, Y. G. (1995) *Arch. Biochem. Biophys. 319*, 23−35.
16. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science 270*, 296−299.
17. Knebel, A., Rahmsdorf, H. J., Ullrich, A., and Herrlich, P. (1996) *EMBO J. 15*, 5314−5325.
18. Hecht, D., and Zick, Y. (1992) *Biochem. Biophys. Res. Commun. 188*, 773−779.
19. Sullivan, S. G., Chiu, D. T., Errasfa, M., Wang, J. M., Qi, J. S., and Stern, A. (1994) *Free Radicals Biol. Med. 16*, 399−403.
20. Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q., and Holbrook, N. J. (1996) *J. Biol. Chem. 271*, 4138−4142.
21. Yan, S. R., and Berton, G. (1996) *J. Biol. Chem. 271*, 23464−23471.
22. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) *J. Biol. Chem. 272*, 217−221.
23. Ahmad, F., Li, P. M., Meyerovitch, J., and Goldstein, B. J. (1995) *J. Biol. Chem. 270*, 20503−20508.
24. Seely, B. L., Staubs, P. A., Reichart, D. R., Berhanu, P., Milarski, K. L., Saltiel, A. R., Kusari, J., and Olefsky, J. M. (1996) *Diabetes 45*, 1379−1385.
25. Ahmad, F., Considine, R. V., and Goldstein, B. J. (1995) *J. Clin. Invest. 95*, 2806−2812.
26. Kulas, D. T., Goldstein, B. J., and Mooney, R. A. (1996) *J. Biol. Chem. 271*, 748−754.
27. Kulas, D. T., Zhang, W. R., Goldstein, B. J., Furlanetto, R. W., and Mooney, R. A. (1995) *J. Biol. Chem. 270*, 2435−2438.
28. Ahmad, F., Goldstein, B. J., Zhang, W. R., Li, P. M., Oswald, M. A., Goldstein, B. J., Kulas, D. T., Zhang, W. R., Goldstein, B. J., Furlanetto, R. W., and Mooney, R. A. (1997) *J. Biol. Chem. 272*, 448−457.
29. Flint, A. J., Tiganis, T., Barford, D., and Tonks, N. K. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1680−1685.
30. Denu, J. M., Zhou, G., Wu, L., Zhao, R., Yuvaniyama, J., Saper, M. A., and Dixon, J. E. (1995) *J. Biol. Chem. 270*, 3796−3803.
31. Lohse, D. L., Denu, J. M., Santoro, N., and Dixon, J. E. (1997) *Biochemistry 36*, 4568−4575.
32. Davies, S. P., Helps, N. R., Cohen, P. T., and Hardie, D. G. (1995) *FEBS Lett. 377*, 421−425.
33. Denu, J. M., Zhou, G., Guo, Y., and Dixon, J. E. (1995) *Biochemistry 34*, 3396−3403.
34. Renart, J., Reiser, J., and Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3116−3120.
35. Denu, J. M., and Dixon, J. E. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 5910−5914.
36. Barford, D., Flint, A. J., and Tonks, N. K. (1994) *Science 263*, 1397−1404.
37. Yuvaniyama, J., Denu, J. M., Dixon, J. E., and Saper, M. A. (1996) *Science 272*, 1328−1331.
38. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z. Y., Dixon, J. E., and Saper, M. A. (1994) *Nature 370*, 571−575.
39. Denu, J. M., Stuckey, J. A., Saper, M. A., and Dixon, J. E. (1996) *Cell 87*, 361−364.
40. Zhou, G., Denu, J. M., Wu, L., and Dixon, J. E. (1994) *J. Biol. Chem. 269*, 28084−28090.
41. Claiborne, A., Miller, H., Parsonage, D., and Ross, R. P. (1993) *FASEB J. 7*, 1483−1490.

42. Ellis, H. R., and Poole, L. B. (1997) *Biochemistry 36*, 15013−15018.
43. Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A., and Dixon, J. E. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2493−2498.
44. Gonzalez-Rubio, M., Voit, S., Rodriguez-Puyol, D., Weber, M., and Marx, M. (1996) *Kidney Int. 50*, 164−173.
45. Krejsa, C. M., Nadler, S. G., Esselstyn, J. M., Kavanagh, T. J., Ledbetter, J. A., and Schieven, G. L. (1997) *J. Biol. Chem. 272*, 11541−11549.
46. Abe, J., Takahashi, M., Ishida, M., Lee, J. D., and Berk, B. C. (1997) *J. Biol. Chem. 272*, 20389−20394.
47. Fialkow, L., Chan, C. K., and Downey, G. P. (1997) *J. Immunol. 158*, 5409−5417.
48. Skorey, K., Ly, H. D., Kelly, J., Hammond, M., Ramachandran, C., Huang, Z., Gresser, M. J., and Wang, Q. (1997) *J. Biol. Chem. 272*, 22472−22480.
49. Huyer, G., Liu, S., Kelly, J., Moffat, J., Payette, P., Kennedy, B., Tsaprailis, G., Gresser, M. J., and Ramachandran, C. (1997) *J. Biol. Chem. 272*, 843−851.
50. Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) *Science 257*, 1496−1502.
51. Yeh, J. I., Claiborne, A., and Hol, W. G. (1996) *Biochemistry 35*, 9951−9957.
52. Poole, L. B. (1996) *Biochemistry 35*, 65−75.
53. Miller, H., and Claiborne, A. (1991) *J. Biol. Chem. 266*, 19342−19350.
54. Lin, W. S., Armstrong, D. A., and Gaucher, G. M. (1975) *Can. J. Biochem. 53*, 298−307.
55. Willett, W. S., and Copley, S. D. (1996) *Chem. Biol. 3*, 851−857.
56. Crane, E. J., Parsonage, D. and Claiborne, A. (1996) *Biochemistry 35*, 2380−2387.
57. Caselli, A., Camici, G., Manao, G., Moneti, G., Pazzagli, L., Cappugi, G., and Ramponi, G. (1994) *J. Biol. Chem. 269*, 24878−24882.
58. Pregel, M. J. and Storer, A. C. (1997) *J. Biol. Chem. 272*, 23552−23558.
59. Storz, G., Tartaglia, L. A., and Ames, B. N. (1990) *Science 248*, 189−194.
60. Wang, X., Culotta, V. C., and Klee, C. B. (1996) *Nature 383*, 434−437.

BI973035T