Roles of Superoxide Radical Anion in Signal Transduction Mediated by Reversible Regulation of Protein-tyrosine Phosphatase 1B*

(Received for publication, September 1, 1999, and in revised form, September 29, 1999)

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Growth factors induce intracellular production of reactive oxygen species in non-phagocytic cells and elevation of their phosphorylated protein tyrosine level. The latter can be achieved by activating protein-tyrosine kinases and/or inactivating protein-tyrosine phosphatases (PTPs). A highly abundant PTP, PTP-1B, is known to be inactivated by oxidation of its catalytic site Cys-215. We show that O2− is kinetically more efficient and chemically more specific oxidant than H2O2 for inactivating PTP-1B. The second-order rate constant for the O2− and H2O2-mediated inactivation is 334 ± 45 M−1 s−1 and 42.8 ± 3.8 M−1 s−1, respectively. PTP-1B oxidized by H2O2 exhibits significantly more oxidized methionine residues and shows a lower degree of reversibility. The initial oxidative product, the Cys-215 sulfenic derivative, can easily be oxidized further to its irreversible sulfenic and sulfonic derivatives. This step is prevented by glutathionylation of the sulfenic derivative to form a glutathionylated PTP-1B, which can be reactivated by dithiothreitol or thioredoxin. Thus, a signal transduction mechanism mediated by the O2− and the participation of glutathione is proposed for the regulation of PTP-1B. This mechanism is supported by the in vivo demonstration that glutathionylated PTP-1B at Cys-215 is formed in A431 cells when they were treated with epidermal growth factor.

Cyclic cascades, which include reversible tyrosine phosphorylation step(s), play a pivotal role in regulating cell cycles and signal transduction due, in part, to their enormous capacity for integrating biological information and for signal amplification (1, 2). Recent findings indicated that treatment with growth factors (3, 4, 8) or H2O2 (5, 6) induces an elevation of tyrosine phosphorylated proteins in non-phagocytic cells. This elevation can be achieved by the activation of protein-tyrosine kinases (PTKs) and/or inactivation of protein-tyrosine phosphatases (PTPs). The latter is particularly important, since PTPs exhibit much higher specific activity relative to that of PTKs (7). Furthermore, reactive oxygen species (ROS), such as O2− and H2O2, have been shown to be transiently generated intracellularly when non-phagocytic cells were stimulated with cytokine or growth factors (3, 4, 8). While there is no convincing evidence to show that the PTKs can be activated by ROS, PTPs have been shown to be regulated by a redox mechanism (9–14). Among them, PTP-1B, a highly abundant PTP, contains a low pKa (about 5.6) active site cysteine 215 (14), making it an ideal specific regulatory site for ROS. Furthermore, Cys-215 is surrounded by main chain amides, which constitute the phosphate binding loop, and the positively charged amino acid residues Arg-45, Arg-47, Lys-116, Lys-120, Arg-112, and Arg-221, likely for attracting the negatively charged phosphate moiety of its substrate (7, 15). Thus, it is reasonable to expect that it will be a favorable target for the negatively charged O2−. In order to identify which ROS, H2O2 or O2−, is the more efficient oxidant in regulating PTP-1B during signaling, we investigated the kinetics of PTP-1B inactivation by each of these oxidants and examined the reversibility of the oxidatively inactivated PTP-1B. Based on the results, a regulatory mechanism was proposed and verified by data obtained from an in vivo study.

EXPERIMENTAL PROCEDURES

Materials and Cell Culture—30% hydrogen peroxide was supplied by Fisher. Chelex 100 Resin (200–400 mesh, sodium form) was from Bio-Rad. Glutathione, manganese superoxide dismutase (Escherichia coli) (MnSOD), DTT, and xanthine (X) were from Sigma. Xanthine oxidase (bovine) (XO) was from Roche Molecular Biochemicals. The synthetic peptide DADEpYLIPQQG (corresponding to EGFR988–998) was from Alpha Diagnostic International (San Antonio, TX). Recombinant PTP-1B was purified as described previously (16). Human A431 epidermoid carcinoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum under 5% CO2. Anti-PTP-1B was supplied by Upstate Biotechnology.

Assay of PTP-1B Activity—The activity of PTP-1B was monitored using continuous spectrophotometric assay described previously (17). Briefly, the peptide substrate was incubated at 25 °C in 50 mM imidazole–HCl, 1 M NaCl, and 60 mM MgCl2 and 60 μM DTPA, pH 7.0, that had been cheled overnight. An aliquot of PTP-1B was added to the substrate and activity was monitored by the increase in fluorescence at 305 nm with an excitation wavelength of 280 nm. Kinetic constants are calculated using a nonlinear least squares with the MLAB program (Civilized Software Inc., Bethesda, MD).

Inactivation of PTP-1B—When PTP-1B was inactivated by O2− the reaction was carried out in either the presence or absence of catalase. 500 nM of PTP-1B was incubated with 2 mM xanthine, 18–36 mM XO, and O2−, which was supplied by bubbling air. Inactivation of PTP-1B by H2O2 was carried out using two methods. One involved incubating 500 nM PTP-1B with H2O2 generated by dismutating the O2•− produced by twice the concentration of XO as described above with 10 μM MnSOD as catalyst. The other involved direct addition of 5–50 μM H2O2 to 500 mM PTP-1B. The second-order rate constant for the latter case was determined by a H2O2 concentration-dependent study. The second-order rate constant for PTP-1B with H2O2 generated by dismutating the O2•− produced by twice the concentration of XO as described above with 10 μM MnSOD as catalyst.

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‡ Both authors supported by National Institutes of Health Grant CA82902.

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* The abbreviations used are: PTK(s), protein-tyrosine kinase(s); PTP(s), protein-tyrosine phosphatase(s); ROS, reactive oxygen species; X, xanthine; XO, xanthine oxidase; MnSOD, manganese superoxide dismutase; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; LC/MS/MS, liquid chromatography/tandem mass spectrometry; EGF, epidermal growth factor; DTT, dithiothreitol; DTPA, diethylenetriaminepentaacetic acid; Cat, catalase.

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constant for the inactivation of PTP-1B by \( \text{O}_2 \) generated by the XO-catalyzed reaction and by \( \text{H}_2\text{O}_2 \) generated from \( \text{O}_2 \) were determined by curve fitting using a combined Adams-Gear method (18) (Civilized Software Inc.) to solve the differential equations required to describe the reaction schemes and the specific activity of XO. In each case, only the inactivating rate constant was treated as a variable. The rate constants used for calculating the formation of \( \text{H}_2\text{O}_2 \) catalyzed by Mn-SOD are those reported by Pick et al. (19).

**Product Analysis via Mass Spectrometry**—In-gel tryptic digests and peptide extractions were performed as described previously (10, 20). Oxidized PTP-1B tryptic fragments appeared as a higher mass of 16 Da or its higher multiple for those containing oxidized cysteine and/or oxidized methionine residue(s), and 305 Da for glutathionylated cysteine, relative to their corresponding unoxidized tryptic fragments. When 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was added to trap the sulfenic derivatives, the resulting fragments exhibited a high mass of 163 Da for the sulfoxide derivative of NBD-Cl and 179 Da for the sulfide derivative of NBD-Cl.

Liquid chromatography/tandem mass spectrometry analysis was performed when additional confirmation of the oxidative modification was required through peptide sequencing as described (10). Fragmentation data (MS/MS) was acquired in centroid format using the data-dependent mode where a preselected set of ions were fragmented after a threshold of at least \( 5 \times 10^5 \) counts (as detected by the quadrupole ion trap liquid chromatography software) is exceeded. The LC/MS/MS analysis was repeated twice for a total of three scans for averaging. The differential in mass due to oxidative modification is similar to the data obtained with the matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF). The mass isolation window for parent ions was 4 Da, the scan range (during parent ion scan) was 5020–2000, and the arbitrary relative collision energy was set to 30%.

**Immunoprecipitation**—PTP-1B was immunoprecipitated with anti-PTP-1B antibody following the recommended protocol with modification. Four \( \mu \)g of anti-PTP-1B was added directly to cell lysates. The mixture was incubated overnight with rocking. The immunocomplex was captured with the addition of 100 \( \mu \)l of protein A-agarose (Upstate Biotechnology) and incubated at 4 °C for 2 h. The beads were collected by pulsed centrifugation and washed three times with cold phosphate-buffered saline. PTP-1B was eluted with a 100 mM glycine solution containing 1.5 mM MgCl\(_2\) at pH 2.65. The protein was then desalted by buffer containing 1.5 M MgCl\(_2\) at pH 2.65. The protein was then desalted by pulsed centrifugation and washed three times with cold phosphate-buffered saline. PTP-1B was eluted with a 100 mM glycine solution containing 1.5 mM MgCl\(_2\) at pH 2.65. The protein was then desalted by pulsed centrifugation and washed three times with cold phosphate-buffered saline.

![Fig. 1. Inactivation of PTP-1B by \( \text{O}_2 \) in the presence of 18 nm XO (bovine), 2 nm xanthine, and \( \text{O}_2 \) supplied by bubbling air (●) and by \( \text{H}_2\text{O}_2 \) under similar conditions as described above, except that the XO concentration was raised to 36 nm and with the addition of 10 \( \mu \)M \( \text{E. coli} \) MnSOD (○). Recombinant PTP-1B (500 nm) was incubated at 25 °C with the \( \text{O}_2 \) or \( \text{H}_2\text{O}_2 \)-generating system in buffer containing 150 mM NaCl, 50 mM imidazole, and 60 \( \mu \)M DTPA at pH 7.0. At the indicated times, the activity was assayed after 25-fold dilution for its ability to dephosphorylate the DADEpYL1PQQG peptide, as monitored by the increase in fluorescence at 305 nm when excited at 280 nm. The lines were calculated with \( k_{\text{inact}} = 334 \text{ M}^{-1}\text{s}^{-1} \) for \( \text{O}_2 \) (solid line) and 42.8 \( \text{M}^{-1}\text{s}^{-1} \) for \( \text{H}_2\text{O}_2 \) (dashed line) inactivation.

**Fig. 3. Quadrupole ion trap liquid chromatography/tandem mass spectrometry (LC/MS/MS) analysis of the triply charged tryptic fragment 28 of PTP-1B containing the active site cysteine, Cys-215. The sample was treated with xanthine oxidase/xanthine followed by NBD-Cl. The data showed that fragment 28 exhibited a higher mass of 179 Da for the sulfoxide derivative of NBD indicating that Cys-215 was in the cysteine sulfenic acid form prior to reacting with NBD-Cl (2354.55 Da).**
Superoxide Radical-mediated Reversible Regulation of PTB-1B

RESULTS AND DISCUSSION

The kinetics of PTB-1B inactivation by H₂O₂ and O₂⁻ was investigated. Fig. 1 shows the time course for the inactivation of PTB-1B by O₂⁻ as it is generated by the XO-catalyzed reaction of X (2 mM) with oxygen and by H₂O₂ generated by twice the concentration of xanthine oxidase in the presence of excess Mn(II)-superoxide dismutase (10 μM). The doubling of the xanthine oxidase for the latter reaction is to account for the fact that two O₂⁻ are needed to form one H₂O₂. It is important to note that in all of the experiments reported here the buffer used was chelaxed overnight, and the enzymes were dialyzed to remove contaminating metal ions, particularly those of iron and copper. The solid line for the O₂⁻-mediated inactivation was calculated using the O₂⁻ inactivation rate constant of 334 ± 45 m⁻¹ s⁻¹. This second-order rate constant was determined by curve fitting using a combined Adams-Gear numerical integration method (18) (Civilized Software Inc.) to solve the differential equations required to describe the reaction scheme. The rate of O₂⁻ production was calculated using the specific activity of XO.

The kinetics of the inactivation was not altered by the presence of excess catalase (data not shown). The second-order rate constant for the PTB-1B inactivation by H₂O₂ was similarly calculated to be 42.8 ± 3.8 m⁻¹ s⁻¹, which was used to obtain the calculated curve shown in Fig. 1. For both curve fittings, only the inactivating rate constant was treated as a variable. The time course for the H₂O₂ production was calculated using the mechanism and rate constants reported for the MnSOD (19). The value for the H₂O₂ inactivation so determined is in good agreement with a value of 43 m⁻¹ s⁻¹ obtained from a H₂O₂ concentration-dependent study by direct H₂O₂ addition (data not shown). This value is somewhat higher than the 7 m⁻¹ s⁻¹ reported for the thiolate ion reacting with H₂O₂ to form its sulfinic derivative (21). The discrepancy can be attributed to the difference in experimental conditions or to the positive effect of the protein structure.

Table I shows that, in addition to the oxidation of Cys-215, some other residues were also found to be oxidized within 30 min of incubation of PTB-1B with either 50 μM H₂O₂, XO/SOD, or XO/Cat in the presence of 2 mM xanthine with the enzyme concentrations similar to those described in the legend of Fig. 1. It is clear that, with H₂O₂ as oxidant, the mass spectrometric analysis revealed that there are more residues, particularly those of methionine, that were oxidized. Fig. 2 shows that when PTB-1B was inactivated to approximately 10% of its total activity by either O₂⁻, the XO/Cat system, or H₂O₂, the XO/SOD system, there is a clear differential in its reversibility induced by DTT. The H₂O₂-inactivated PTB-1B shows only a 20% recovery, while the O₂⁻-inactivated enzyme recovered about 60–65% of its activity.

Since the reversible sulfenic derivative, produced initially, can easily be oxidized to form its irreversible sulfonic and sulfenic derivatives, it is necessary to trap the sulfenic derivative in order to verify its formation by mass spectrometric method. The NBD-Cl was used to trap the cysteine sulfenic derivative after oxidation of PTB-1B. NBD-Cl has been shown to react with sulfhydryls and sulfenic acids of cysteine to form a thioether and sulfoxide derivative, respectively (22). The mass difference of 16 Da between the thioether and sulfoxide derivative, respectively (22). The mass difference of 16 Da between the two derivatives can be distinguished readily by mass spectrometry. Fig. 3 shows the mass spectrum obtained for the quadrupole ion trap LC/MS/MS of the tryptic fragment of the O₂⁻-oxidized PTB-1B, which contains the active site Cys-215. The fragments b₁₋₁₀, b₁₋₁₂, y₁₋₁₂, y₁₋₁₇, y₁₋₁₈, y₁₋₁₉, y₁₋₂₀, and y₁₋₂₁ all contain the NBD-sulfoxide derivative of Cys-215. The b₁₋₁₀, b₁₋₁₂, b₁₋₁₄, b₁₋₁₆, b₁₋₁₈, and b₁₋₂₀ fragments do not contain the modifying group. A similar spectrum was also obtained for the same tryptic fragment derived from the NBD-Cl-treated H₂O₂-oxidized PTB-1B. Thus, both O₂⁻ and H₂O₂ are capable of oxidizing the Cys-215 to its reversible sulfenic derivative.

Glutathionylation of the sulfenic derivative, which can be achieved by the addition of GSH to the O₂⁻-oxidized PTB-1B, will convert it to a relatively more stable inactive phosphatase. Using a mass spectrometric method, we showed that when PTB-1B was oxidized by O₂⁻ and then reacted with 10 mM glutathione, a mixed disulfide was formed between Cys-215 and GSH (data not shown). The glutathionylated PTB-1B may
then be reactivated by the addition of thiols or enzymatically by thioredoxin. The latter can provide an efficient and selective reactivation mechanism (23). Thus, a plausible in vivo mechanism is proposed (Fig. 4) for the signal transduction pathway involving O$_2^\bullet$-mediated inactivation and glutathionylation of PTP-1B.

The validity of the proposed mechanism was tested by monitoring the effect of epidermal growth factor (EGF) on the PTP-1B in A431 human epidermoid carcinoma cells. It had been previously shown (4) that addition of EGF to serum-starved A431 cells resulted in a transient increase in the ROS concentration and the levels of tyrosine-phosphorylated proteins. EGF was incubated with serum-starved A431 cells for 20 min, and the cells were harvested and lysed. PTP-1B was immunoprecipitated with 4 μg of anti-PTP-1B antibody, following the recommended protocol provided by Upstate Biotechnologies, and eluted from the antibody with a 100 mM glycine solution containing 1.5 mM MgCl$_2$ at pH 2.65 prior to electrophoresis on an SDS-polyacrylamide gel electrophoresis gel. The PTP-1B band was then excised and subjected to in-gel trypptic digest similar to the in vitro experiments described above. The sample was analyzed by LC/MS/MS for any Cys-215 glutathionylated PTP-1B, which will yield a mass change of 305 Da. Fig. 5 shows a mass spectrum obtained using this procedure. It clearly reveals the presence of a glutathionylated peptide that contains Cys-215. The fragments $y_7^{+1e}$, $y_8^{+1e}$, $y_9^{+2e}$, $y_10^{+2e}$, and $b_{16}^{+3e}$ all contain a glutathionylated Cys-215 residue, while fragments $b_5^{+1e}$ and $b_6^{+1e}$ do not contain the modified Cys-215. Furthermore, fragments $y_{11}^{+1e}$ and $b_{23}^{+3e}$ contain the modifications but have lost the glycine residue from the fragmentation of glutathione itself. Fragment $y_{13}^{+2e}$ contains the glutathionylated fragment but has lost the glutamate residue of glutathione. In the control experiment performed with identical procedures except that no EGF was added, no glutathionylated PTP-1B was observed (data not shown).

Our data clearly show that relative to H$_2$O$_2$, O$_2^\bullet$ is kinetically and chemically a more efficient regulator for PTP-1B. It initially forms an unstable sulfenic derivative at the active site Cys-215 of the phosphatase. The inactivation likely proceeds via the following mechanism in the presence of oxygen and GSH,

$$\text{RS}^- + \text{O}_2^\bullet + \text{H}^+ \rightarrow \text{RSO}^- + \text{OH}^- \quad \text{(Reaction 1)}$$

$$\text{RSO}^- + \text{GSH} \rightarrow \text{GS}^- + \text{RSOH} \quad \text{(Reaction 2)}$$

$$\text{GS}^- + \text{GSH} \rightarrow \text{GSSG}^- + \text{H}^+ \quad \text{(Reaction 3)}$$

$$\text{GSSG}^- + \text{O}_2 \rightarrow \text{GSSG} + \text{O}_2^\bullet \quad \text{(Reaction 4)}$$

$$\text{RSOH} + \text{GSH} \rightarrow \text{RSSG} + \text{H}_2\text{O} \quad \text{(Reaction 5)}$$

where RS$^-$ and RSO$^-$ represent the protein sulfhydryl and its sulfenic derivative, respectively. In this reaction scheme, the GS$^-$ and RSO$^-$ radicals are intermediates or chain carriers, both of which were detected and described in our previous reports (24, 25), and the formation of RSOH and RSSG was verified by the current results. The rate constant, 334 m$^{-1}$ s$^{-1}$, for O$_2^\bullet$ inactivation of PTP-1B is significantly higher than that of H$_2$O$_2$, which is 43 m$^{-1}$ s$^{-1}$. If the ROS generated due to EGF binding is O$_2^\bullet$ and two O$_2^\bullet$ are required to form one H$_2$O$_2$, this should further enhance the kinetic advantage of O$_2^\bullet$ over H$_2$O$_2$. Furthermore, O$_2^\bullet$ should be more specific than the neutral H$_2$O$_2$ when the site of action is surrounded by positively charged residues, like the catalytic site of PTP-1B. In addition, the O$_2^\bullet$-inactivated PTP-1B is more reversible than that of H$_2$O$_2$, due to the fact that significantly more methionine residues are oxidized by H$_2$O$_2$. Since the sulfenic derivative can easily be oxidized to form the irreversible sulfenic and sulfonic products, we show that in the presence of GSH, the sulfenic derivative is converted to a more stable $S$-glutathionylated PTP-1B, which can be reactivated by either DTT or thioredoxin. Thus, it provides an efficient regulatory mechanism for the regulation of PTP-1B in signal transduction. The proposed regulatory mechanism is supported by the observation that glutathionylated PTP-1B at Cys-215 is formed in A431 cells when they were treated with EGF. Since PTP active sites share similar structural features (26), the proposed regulatory mechanism will likely apply to the whole PTP family.

**Acknowledgments**—We thank Drs. J. Michael Poston, Henry Fales, and Simone Konig (NHLLB) and Dr. David Davis (NCI) for helpful discussions. We also thank Dr. Chien-Chung Chao for helping with the preparation of A431 cells.

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doi: 10.1074/jbc.274.49.34543

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