Biochimica et Biophysica Acta 1834 (2013) 464–469

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Redox regulation of protein tyrosine phosphatase activity by hydroxyl radical☆

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ARTICLE INFO

Article history:
Received 20 April 2012
Received in revised form 25 June 2012
Accepted 30 June 2012
Available online 20 July 2012

Keywords:
Protein tyrosine phosphatase
Hydrogen peroxide
Hydroxyl radical
Redox regulation

ABSTRACT

Substantial evidence suggests that transient production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) is an important signaling event triggered by the activation of various cell surface receptors. Major targets of H_2O_2 include protein tyrosine phosphatases (PTPs). Oxidation of the active site Cys by H_2O_2 abrogates PTP catalytic activity, thereby potentially furnishing a mechanism to ensure optimal tyrosine phosphorylation in response to a variety of physiological stimuli. Unfortunately, H_2O_2 is poorly reactive in chemical terms and the second order rate constants for the H_2O_2-mediated PTP inactivation are ~10 M^{-1} s^{-1}, which is too slow to be compatible with the transient signaling events occurring at the physiological concentrations of H_2O_2. We find that hydroxyl radical is produced from H_2O_2 solutions in the absence of metal chelating agent by the Fenton reaction. We show that the hydroxyl radical is capable of inactivating the PTPs and the inactivation is active site directed, through oxidation of the catalytic Cys to sulfenic acid, which can be reduced by low molecular weight thiols. We also show that hydroxyl radical is a kinetically more efficient oxidant than H_2O_2 for inactivating the PTPs. The second-order rate constants for the hydroxyl radical-mediated PTP inactivation are at least 2–3 orders of magnitude higher than those mediated by H_2O_2 under the same conditions. Thus, hydroxyl radical generated *in vivo* may serve as a more physiologically relevant oxidizing agent for PTP inactivation. This article is part of a Special Issue entitled: Chemistry and mechanism of phosphatases, diesterases and triesterases.

1. Introduction

Protein tyrosine phosphorylation plays a central role in many cellular processes ranging from growth and metabolism to adhesion and differentiation [1]. Major insights into tyrosine phosphorylation mediated cellular events have been derived from studies of protein tyrosine kinases (PTKs). This is due in part to the fact that many transmembrane receptors for peptide hormones and growth factors possess intrinsic PTK activity. Receptors for cytokines lack intrinsic kinase activity but associate with non-receptor PTKs inside the cell. Consequently it is common to view signaling pathways as cascades of reactions emanating from the PTKs. However, protein tyrosine phosphorylation is a dynamic process controlled by two opposing biochemical reactions catalyzed by PTK and protein tyrosine phosphatases (PTP) [2]. Not surprisingly, the disturbance of the normal balance between PTK and PTP activity leads to aberrant tyrosine phosphorylation, which has been linked to a variety of human diseases. Given the reversible nature of protein tyrosine phosphorylation, illumination of the regulatory mechanisms for PTP function is a prerequisite to gaining a complete understanding of the physiological consequences of tyrosine phosphorylation and how such signaling events are abrogated in pathological conditions.

An emerging layer of regulation of the PTP activity is reversible inactivation through stimulus-mediated oxidation. Although initially viewed as toxic byproducts, reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) are increasingly recognized as important regulators of cellular and physiological processes [3,4]. For example, stimulation of cognate cell surface receptors with ligands as diverse as insulin [5], EGF [6], PDGF [7] or TGF-β [8] induces a burst of intracellular production of H_2O_2 immediately after ligand binding. Importantly, it appears that H_2O_2 production is required for ligand-mediated PTK activation and tyrosine phosphorylation. A major proposed mechanism by which the ligand-induced H_2O_2 regulates the tyrosine phosphorylation-dependent signaling is through transient oxidation and inactivation of the PTPs [9–13].

The PTPs constitute a large family of enzymes (>100) that parallel PTKs in their structural diversity and complexity [14]. Unlike protein kinases, where tyrosine specific and serine/threonine specific kinases share sequence identity, the PTPs show no sequence similarity with serine/threonine phosphatases, or the broad specificity phosphatases such as acid or alkaline phosphatases. The hallmark that defines the PTP superfamily is the active site sequence (I/V)H**C**XAGXG**R**(S/T), the PTP signature motif. Extensive mechanistic studies have established that members of the PTP family utilize a common mechanism for substrate turnover [15]. A key feature in the PTP catalytic

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doi:10.1016/j.bbapap.2012.06.018

mechanism is the utilization of the active site cysteine as the attacking nucleophile to form a thiophosphoryl enzyme intermediate, which is hydrolyzed in the dephosphorylation reaction. Due to the highly positively charged environment in the PTP active site, the sulfhydryl group of this Cys residue has an extremely low pKa (~5) [16,17] in comparison to the typical pKa for a Cys residue in proteins (~8.5). Thus at physiological conditions, the side chain of the PTP active site Cys exists as the thiolate anion, which not only enhances its nucleophilicity but also renders it susceptible to oxidation. Biochemical studies indicate that upon exposure to H_2O_2, the catalytic Cys is converted into the sulfenic acid form (Cys-SOH), effectively inactivating the PTPs because the oxidized Cys can no longer function as a nucleophile [9,11,18]. This offers a biochemical basis for why ROS generation enhances growth factor-induced tyrosine phosphorylation. The sulfenic acid can be reduced back to the thiolate form through the action of cellular thiols [19], leading to the restoration of PTP activity. Thus, oxidation of the catalytic Cys by ROS is reversible and represents a dynamic mechanism of PTP regulation.

H_2O_2, being produced in cells downstream of many surface receptors, is thought to be the major ROS for the reversible oxidation of sulfhydryl groups inside the cell. Indeed, the majority of studies examining PTP redox regulation have been performed with H_2O_2. However, although H_2O_2 is capable of oxidizing the PTPs, H_2O_2 is poorly reactive in chemical terms [20]. The rate constants measured for H_2O_2-mediated PTP inactivation are in the range of 10–20 M^{-1} s^{-1} [9,21], indicating that the loss of PTP activity will be a very slow process ($t_{1/2}$ > 10 h) at the physiological concentrations of H_2O_2 thought to exist during signaling events (0.1–1 μM) [22,23]. Paradoxically, ROS-mediated PTP inactivation during signaling events typically occurs rapidly (2–5 min) which coincides with the transient increase in H_2O_2 concentration upon growth factor stimulation [5,7,10,13]. The kinetic discrepancy between the observed robust PTP inactivation during cellular signaling events and the apparent sluggish activity of H_2O_2 toward the PTPs in the test tube may be reconciled if H_2O_2 undergoes spontaneous or enzymatic conversion to more reactive oxidizing agents that can mediate rapid intracellular PTP inactivation [22–24]. In this study we provide evidence that hydroxyl radical may serve as the more physiologically relevant oxidizing agent for PTP inactivation.

2. Materials and methods

2.1. Materials

Catalase (from bovine liver), superoxide dismutase (from *E. coli*), glutathione (GSH, 98% purity), and 30% H_2O_2 solution were from Sigma; 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl, 98% purity), 5,5-Dimethyl-1-pyrroline N-oxide (DMPO, 97% purity) were Aldrich products; and DTT was from LabScientific Inc. $FeCl_2$ and $MnCl_2$ were obtained from Sigma-Aldrich.

2.2. Inactivation assay

All PTP inactivation reactions were measured continually by following the change of optical density at 405 nm at 25 °C. The reaction was initiated by adding enzyme to the 1 ml reaction system in a cuvette: 50 mM 3,3-dimethylglutarate (pH 6.0), 20 mM *p*-nitrophenyl phosphate (*p*NPP), H_2O_2 of different concentrations, with or without 1 mM EDTA. The final enzyme concentrations were 10 nM for PTP1B, 80 nM for VHR, 32 nM for PTPα, 40 nM for HePTP, and 30 nM for CD45. The observed first-order inactivation rate constant k, was obtained by fitting the data to $Abs = (Amp)e^{-kt} + B$, where Abs is absorbance at 405 nm, Amp is the change in absorbance, k is the first-order rate constant, t is time, and B is the starting absorption [9]. The second-order rate constant for the inactivation in the presence of EDTA, was obtained by plotting the first-order rate constant versus H_2O_2 concentration; the second-order rate constant for the inactivation in the absence of EDTA, was derived from fitting the first order rate constant to the Michaelis–Menten formulation.

2.3. NBD-Cl modification of PTP1B

10 μM PTP1B in 50 mM 3,3-dimethylglutarate buffer (pH 6.0) was firstly treated with 1 mM DTT to fully reduce all cysteine residues. The DTT was then removed by Amicon ultra PL-10 (Molecular weight cut-off is 10,000, from Amersham Pharmacia). H_2O_2 was added to the final concentration of 300 μM for the oxidation of PTP1B in the presence of 1 mM EDTA and 30 μM for the oxidation of PTP1B in the absence of EDTA. After 15 min, 100 units of catalase was added to eliminate the residual H_2O_2. After 3 min catalase treatment, NBD-Cl was added to a final concentration of 0.6 mM. The modification reactions were then allowed to proceed at room temperature for 30 min. The samples were then spinned with Amicon ultra PL-10 with multiple changes of the 50 mM 3,3-dimethylglutarate buffer (pH 6.0) to remove the residual NBD-Cl. Finally, the sample with a final volume of 0.5 ml was scanned by a UV spectrometer [9].

2.4. EPR measurements

The following buffer solutions were freshly made: 50 mM 3,3-dimethylglutarate pH 6.0, without EDTA; 50 mM 3,3-dimethylglutarate pH 6.0, 1 mM EDTA; 20 mM PBS, pH 6.0, without EDTA; 20 mM PBS, pH 6.0, 1 mM EDTA. A stock of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) was prepared in water (880 mM). DMPO was added to an appropriate buffer with or without 100 μM H_2O_2 to a final concentration of 88 mM. 50 μl of the incubation mixture was immediately sealed in a capillary tube before the EPR measurement. EPR spectra were obtained with a Bruker 200D X-band spectrometer with ESP 300 upgrade and VT 4111 temperature controller. Instrumental settings were as follows: temperature, 25 °C; microwave power, 20 mW; microwave frequency, 9.514 GHz; modulation amplitude, 1.011 G, and receiver gain, 1.60×10^5.

3. Results and discussion

The term ROS encompasses many species including H_2O_2, superoxide anion radical, lipid peroxides, nitric oxide and hydroxyl radical. In addition to H_2O_2, it has been shown that treatment of various PTPs with superoxide radical anion [11], nitric oxide [25], and lipid peroxides [26] all lead to the oxidation of the active site Cys. The majority of studies have analyzed the effects of H_2O_2 since it is produced upon activation of many cell surface receptors. However, the concept that H_2O_2 is the most relevant oxidant for the PTPs has been recently challenged based upon kinetic considerations of H_2O_2 reactivity. Thus, the exact identity of the oxidants which directly mediate PTP oxidation is not known. Although H_2O_2 is not active, it can be readily converted to much more reactive hydroxyl radical (•OH), either by exposure to UV light or by reduced (lower oxidation states) transition metal ions through the Fenton reaction [20,27]. In the following we provide evidence that hydroxyl radical (•OH) is highly reactive with the PTP and may serve as a physiologically relevant PTP oxidant.

3.1. PTPs can be more rapidly inactivated by H_2O_2 in the absence of EDTA

The potential for hydroxyl radical as a physiological oxidant for the PTPs has not been considered. This may have stemmed from the fact that in kinetic studies it is a common practice to include EDTA or other chelating agents in the assay buffers in order to protect the active site thiols of the PTPs from reacting with heavy metals as well as metal ion-catalyzed oxidation by molecular O_2. We hypothesized that in the absence of EDTA, the rate of H_2O_2-mediated PTP inactivation may be accelerated due to hydroxyl radical production from H_2O_2 in the presence of trace amount of free transition metal ions. To test this hypothesis, we initially focused our study on the