Protection of a single-cysteine redox switch from oxidative destruction: On the functional role of sulfenyl amide formation in the redox-regulated enzyme PTP1B

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Model reactions offer a chemical mechanism by which formation of a sulfenyl amide residue at the active site of the redox-regulated protein tyrosine phosphatase PTP1B protects the cysteine redox switch in this enzyme against irreversible oxidative destruction. The results suggest that ‘overoxidation’ of the sulfenyl amide redox switch to the sulfinyl amide in proteins is a chemically reversible event, because the sulfinyl amide can be easily returned to the native cysteine thiol residue via reactions with cellular thiols.

Intracellular concentrations of hydrogen peroxide (H₂O₂) increase under conditions of oxidative stress and during some normal signal transduction processes.¹⁻³ An important mechanism by which cells issue temporary responses to such transitory increases in H₂O₂ levels involves reversible oxidation of cysteine residues on critical ‘sensor’ proteins.⁴,⁵ The ability of cysteine residues to serve as reversible redox switches relies upon the unique ability of the γ-sulfur atom in this amino acid to cycle easily between (at least) two oxidation states under physiological conditions. Specifically, oxidation of a cysteine thiol by H₂O₂ yields a sulfenic acid residue (reaction i, Scheme 1A) that can, over time, be returned to the native thiol by reactions with biological thiols (reaction ii, Scheme 1A).⁴⁻⁸

Protein sulfenic acid residues also have the potential to undergo further reaction with hydrogen peroxide to generate the corresponding sulfinic acid (reaction iii, Scheme 1A).⁴⁻⁹ This reaction is irreversible, except in the case of some peroxiredoxins⁸ and, therefore, yields an overoxidized, ‘broken’ redox switch. Alternatively, in some proteins, the initially-formed sulfenic acid intermediate undergoes reaction with a neighboring ‘back door’ cysteine thiol to generate a disulfide linkage (reaction ii, Scheme 1B).¹⁰⁻¹³ Rudolph and Sohn provided evidence that, at least in the context of the phosphatase Cdc25B, disulfide formation protects the enzyme against irreversible overoxidation.¹⁰,¹¹ There are at least two possible mechanisms underlying this protection. First, the disulfide may be relatively resistant to further oxidation (reaction iii, Scheme 1B).¹⁰,¹¹ Second, if ‘overoxidation’ does occur, the resulting thiosulfinate likely could be converted cleanly back to the native cysteine residues by reactions with biological thiols (reaction v, Scheme 1B).¹⁴

Protein tyrosine phosphatases (PTPs) are important targets of intracellular H₂O₂.¹⁵⁻¹⁷ These cysteine-dependent enzymes catalyze the removal of phosphoryl groups from tyrosine residues on their protein substrates.¹⁵⁻¹⁷ Accordingly, PTPs work in tandem with protein tyrosine kinases to regulate critical signal transduction cascades by modulating the phosphorylation status and, in turn, the functional properties of proteins involved in these pathways.¹⁵⁻¹⁷ The catalytic activity of some PTPs is subject to redox

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regulation as part of normal cell signaling processes. For example, the enzyme PTP1B, a major negative regulator of the insulin signaling pathway, is inactivated by a burst of H₂O₂ that is produced upon binding of insulin to its cell-surface receptor. Subsequent reactions with cellular thiol rapidly follow the enzyme to its active form. This transient oxidative inactivation of PTP1B serves as a ‘timing device’ that increases phosphorylation levels on the insulin receptor and insulin receptor substrates, thus potentiating cellular responses for a defined period of time following insulin stimulation.

For some time, it was widely assumed that redox regulation of PTPs involved either sulfenic acid or disulfide intermediates, as shown in Scheme 1. However, recent studies in the context of PTP1B revealed a new mechanism for redox regulation of PTP activity in which the initially-formed sulfenic acid undergoes reaction with the neighboring amide nitrogen to yield a cyclic sulfenyl amide (known, more formally, as an isothiazolidin-3-one, reaction ii, Scheme 2). As required for a functional redox switch, reactions with biological thiols can convert the sulfenyl amide back to the catalytically active thiol form of the enzyme (reaction iii, Scheme 2). Importantly, sulfenyl amide formation subsequently has been observed in other proteins and it has been suggested that this posttranslational cysteine modification can occur in cells.

Like other cysteine-based redox switches, the sulfenyl amide residue has the potential to undergo ‘overoxidation’ to sulfenyl and sulfonyl derivatives (reactions iv and vii, Scheme 2). Therefore, complete understanding of this redox switch requires consideration of the reactivity of these higher oxidation states under physiologically relevant conditions. In the work described here we employed small organic molecules to model the reactivity of the protein sulfenyl amide residue found in PTP1B. For these studies, it may be important that the pKₐ of the corresponding disulfide in this reaction.

The present studies build upon our previous use of compound 1 to model the reactivity of the protein sulfenyl amide residue found in PTP1B. For these studies, it may be important that the pKₐ of the corresponding disulfide (Scheme 2). In the absence of thiol, the sulfenyl amide residue has the potential to undergo ‘overoxidation’ to sulfenic acid and disulfide intermediates shown in Scheme 4. Consistent with this idea, HPLC analysis at early times in the reaction between 2 and 2-mercaptoethanol (500 μM) in sodium phosphate buffer (pH 7, containing 30% acetonitrile by volume) revealed an intermediate whose retention time and mass match that of the disulfide 4 at m/z 316 for (M+H)+ (Fig. 1). Further, when the reaction was conducted using only two equivalents of thiol, HPLC analysis showed the disulfide to be a major final product alongside unreacted starting material and 5. The thiol, 2-mercaptoethanol, is converted to the corresponding disulfide in this reaction.

The reaction of 2 with excess thiol (1 mM) in sodium phosphate buffer (pH 7, containing 50% acetonitrile by volume) occurs with a pseudo-first-order rate constant of 5.5 ± 0.2 × 10⁻³ s⁻¹ (t₁/₂ ≈ 2 min). This corresponds to an apparent second-order rate constant of 5.5 ± 0.2 M⁻¹ s⁻¹ (Fig. 2). These results offer the prediction that ‘overoxidation’ of the sulfenyl amide redox switch to the sulfenyl amide in proteins is a chemically reversible event, because the sulfenyl amide can be easily returned to the native cysteine thiol residue via reactions with thiols (reaction v, Scheme 2).

In the absence of thiol, the sulfenyl amide 2 undergoes a relatively slow reaction with water to yield the corresponding sulfenic acid derivative 6 in 88% yield (Scheme 5). Compound 6 was characterized as its methyl ester derivative 7 following treatment of the reaction mixture with excess methyl iodide. The hydrolysis of 2 (50 μM) in sodium phosphate buffer (50 mM, pH 7, containing 50% acetonitrile by volume) occurs with a pseudo-first-order rate constant of 4.5 ± 0.2 × 10⁻⁴ s⁻¹, corresponding to a half-life of 26 min (Fig. 3). In contrast, the parent sulfenyl amide 1 is stable under these conditions (no significant decomposition observed over 24 h). These results forecast that protein sulfenyl amide residues can undergo chemically irreversible hydrolysis to the sulfenic acid (reaction vi, Scheme 2); however, rate measurements in the context of this model compound suggest that if water and physiological concentrations of thiol (1–10 mM) enjoy equal access to the sulfenyl amide, thiol-mediated recovery of enzyme activity will be approximately 10–100 times faster than irreversible loss of activity.
10.2 min was identified as the mixed disulfide (275 L), 2-mercaptoethanol (25 L) from the reaction mixture were injected onto a C-18 column (500 mm, pH 7.0), water (150 L), 2-mercaptoethanol (50 L of a 10 mM stock in water), and acetonitrile (247.5 L) at 25 °C. The mixture (final concentrations: 2, 50 mM; buffer, 50 mM, pH 7.0; thiol, 1 mM; acetonitrile, 50% by volume) was vortex mixed and the disappearance of 2 (a is the peak area at time = t and a0 is the peak area at time = 0) monitored by reverse phase HPLC at regular time intervals as described in the legend for Figure 1. From the slope of the plot, a pseudo-first-order rate constant of 5.5 ± 0.2 M⁻¹ s⁻¹ (t1/2 = 2 min) at 1 mM thiol was obtained. This corresponds to an apparent second-order rate constant of 5.5 ± 0.2 M⁻¹ s⁻¹.

Finally, we examined the reactivity of the sulfonyl amide 3. HPLC analysis revealed that this compound is stable in aqueous solution composed of sodium phosphate buffer (50 mM, pH 7.0), water (200 L) and acetonitrile (247.5 L). The mixture (final concentrations: 2, 50 mM; buffer, 50 mM, pH 7.0; acetonitrile, 50% by volume) was vortex mixed and the disappearance of compound 2 (a is the peak area at time = t and a0 is the peak area at time = 0) analyzed by reverse phase HPLC as described in the legend of Figure 1. The pseudo-first-order rate constant of 0.027 ± 0.001 min⁻¹ was obtained from the slope of the plot. This corresponds to a half-life of 26 min for the hydrolysis of 2 under these conditions.

Figure 2. A representative plot for the disappearance of 2 in the presence of thiol. Compound 2 (2.5 µL of a 10 mM stock in CH₃CN) was added to a mixture containing sodium phosphate buffer (50 µL, 500 mM, pH 7.0), water (150 µL), 2-mercaptoethanol (50 µL of a 10 mM stock) and acetonitrile (247.5 µL). The mixture (final concentrations: 2, 50 µM; buffer, 50 mM, pH 7.0; thiol, 1 mM; acetonitrile, 50% by volume) was vortex mixed and the disappearance of 2 (a is the peak area at time = t and a0 is the peak area at time = 0) monitored by reverse phase HPLC at regular time intervals as described in the legend for Figure 1. From the slope of the plot, a pseudo-first-order rate constant of 5.5 × 10⁻³ s⁻¹ (t1/2 = 2 min) at 1 mM thiol was obtained. This corresponds to an apparent second-order rate constant of 5.5 ± 0.2 M⁻¹ s⁻¹.

Figure 3. Representative plot for the disappearance of 2 in the absence of thiol. Compound 2 (2.5 µL of a 10 mM stock in CH₃CN) was incubated at 25 °C in a solution composed of sodium phosphate buffer (50 µL, 500 mM, pH 7.0), water (200 µL) and acetonitrile (247.5 µL). The mixture (final concentrations: 2, 50 µM; buffer, 50 mM, pH 7.0; acetonitrile, 50% by volume) was vortex mixed and the disappearance of compound 2 (a is the peak area at time = t and a0 is the peak area at time = 0) analyzed by reverse phase HPLC as described in the legend of Figure 1. The pseudo-first-order rate constant of 0.027 ± 0.001 min⁻¹ was obtained from the slope of the plot. This corresponds to a half-life of 26 min for the hydrolysis of 2 under these conditions.

Due to hydrolysis. It is interesting to note that thiosulfonates may be similarly labile to hydrolysis. 26,27

Finally, we examined the reactivity of the sulfonyl amide 3. 38 HPLC analysis revealed that this compound is stable in aqueous sodium phosphate buffer (50 mM, pH 7, containing 40% acetonitrile by volume) either in the presence or absence of the thiol, 2-mercaptoethanol. This suggests that exhaustive oxidation of the sulfonyl amide to the sulfonyl amide (Scheme 2, reaction vii) likely represents chemically irreversible destruction of the sulfonyl amide redox switch.

In conclusion, these studies offer chemical insight regarding possible functional roles of sulfonyl amide formation in redox-switched proteins. Sulfonyl amide formation has the potential to protect a single-cysteine redox switch against irreversible oxidation in much the same way that disulfide formation protects dithiol redox switches from oxidative destruction. In both cases, further oxidation to the sulfinyl form yields an intermediate that readily can be resolved by reactions with biological thiols to regenerate the native cysteine residues (Scheme 6). Thus, irreversible oxidative destruction of these switches requires conversion to the sulfonyl derivatives which presumably requires relatively harsh oxidizing conditions (Scheme 6). In contrast, a sulfenic acid redox switch has no failsafe mechanism. The sulfenic acid group readily can be resolved by reactions with biological thiols to regenerate the native cysteine residues (Scheme 6). Thus, irreversible oxidative destruction of these switches requires conversion to the sulfonyl derivatives which presumably requires relatively harsh oxidizing conditions (Scheme 6). In contrast, a sulfenic acid redox switch has no failsafe mechanism. The sulfenic acid group...
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References and notes


19. Synthesis of ethyl 2-(3-oxobenzyl)[3-isothiazol-2(3H)-yl]acetate (1). To a stirred solution of 1H NMR (CDCl3, 300 MHz) δ 1.93 (3H, s), 2.04 (3H, s), 4.25 (4H, m), 6.84 (2H, m), 7.62 (1H, d, J = 7.65 Hz), 7.80 (1H, d, J = 7.65 Hz). 13C NMR (CDCl3, 75.4 MHz) δ 16.97, 168.05, 168.39, 136.12, 131.72, 130.53, 129.66, 128.61, 61.75, 45.16, 41.95, 14.9. HRMS (ESI) calcd for C14H20N2O2S [M+H]+ 286.0754, found 286.0755.

20. Synthesis of ethyl 2-(3-oxobenzyl)[3-isothiazol-2(3H)-yl]acetate (1). A solution of ethyl 2-(3-oxobenzyl)[3-isothiazol-2(3H)-yl]acetate (1) in acetonitrile (632.4 mL) was added to a dry flask under nitrogen. To this was added triethylamine (10 mol %, 14 mL) and the mixture was allowed to stir at room temperature for 2 h. The reaction mixture was monitored by thin layer chromatography and additional aliquots of triethylamine (14 mL) were added every 2 h until all starting material was consumed. When the reaction was complete, the mixture was dried by rotary evaporation, the residue taken up in water (15 mL), and the pH adjusted to 5. This mixture was extracted with ethyl acetate (3 × 15 mL), the combined organic layers dried over sodium sulfate, filtered, and evaporated under reduced pressure. The resulting product was derivatized without purification by addition of methyl iodide (1 mL), followed by stirring at 25 °C for 4 h and acidified to pH 7 using dilute hydrochloric acid. The aqueous solution was extracted with diethyl ether (3 × 5 mL), the combined organic extract washed with water, dried over anhydrous sodium sulfate, filtered, and then evaporated to yield a colorless oil that was purified by flash column chromatography (7:3 ethyl acetate/hexane) to obtain 5 (11 mg, 80%). Rf = 0.61 (3:2 ethyl acetate/hexane). 1H NMR (CDCl3, 300 MHz) δ 1.32 (3H, t, J = 7 Hz), 4.24 (4H, m), 4.74 (1H, s), 6.57 (1H, br, s), 7.17 (1H, m), 7.31 (2H, m), 7.53 (1H, dd, J = 7.65 Hz, 1.0 Hz). 13C NMR (CDCl3, 75.4 MHz) δ 169.79, 168.45, 133.17, 132.16, 131.07, 130.94, 128.12, 125.21, 121.57, 121.43, 15.41, 11.41. HRMS (ESI) calcd for C14H20N2O2S+ [M+H]+ 254.0487, found 254.0493.

21. Reactions of compound 2 with 2-mercaptopentanoic acid in aqueous buffer. To a stirred solution of 2 (15 mg, 0.059 mmol) in acetonitrile (1.78 mL) was added a mixture of sodium phosphate buffer (3.56 mL, 500 mM, pH 7.0), water (538 μL) and 2-mercaptopentanoic acid (622.2 μL of a 14.3 M solution in water). The resulting colorless solution was stirred at 25 °C (final concentrations: 2, 10 mM; buffer, 300 mM, pH 7.0; thiol, 150 mM; acetone, 30% by volume). The starting material disappeared in less than 5 min as indicated by the TLC. The reaction mixture was dried on silica gel and incubated in pH 7 buffer (200 mM, pH 7). 1H NMR (CDCl3, 300 MHz) δ 1.30 (3H, t, J = 7 Hz), 4.24 (4H, m), 4.74 (1H, s), 6.57 (1H, br, s), 7.17 (1H, m), 7.31 (2H, m), 7.53 (1H, dd, J = 7.65 Hz, 1.0 Hz). 13C NMR (CDCl3, 75.4 MHz) δ 169.79, 168.45, 133.17, 132.16, 131.07, 130.94, 128.12, 125.21, 121.57, 121.43, 15.41, 11.41. HRMS (ESI) calcd for C14H20N2O2S+ [M+H]+ 240.0706, found 240.0708.