Biochimica et Biophysica Acta 1852 (2015) 905–912

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Peroxide-mediated oxidation and inhibition of the peptidyl-prolyl isomerase Pin1

Brendan T. Innes [a,1], Modupeola A. Sowole [b], Laszlo Gyenis [a], Michelle Dubinsky [a], Lars Konermann [a,b], David W. Litchfield [a,c], Christopher J. Brandl [a], Brian H. Shilton [a,*]

[a] Department of Biochemistry, Schulich School of Medicine and Dentistry, The University of Western Ontario, Canada
[b] Department of Chemistry, The University of Western Ontario, Canada
[c] Department of Oncology, Schulich School of Medicine and Dentistry, The University of Western Ontario, Canada

ARTICLE INFO

Article history:
Received 9 July 2014
Received in revised form 18 December 2014
Accepted 23 December 2014
Available online 13 January 2015

Keywords:
Peptidyl-prolyl *cis–trans* isomerase
Oxidation
Cysteine sulfenic and sulfinic acid
Alzheimer's disease
Kinase signaling
Cancer biology

ABSTRACT

Pin1 is a phosphorylation-dependent peptidyl-prolyl isomerase that plays a critical role in mediating protein conformational changes involved in signaling processes related to cell cycle control. Pin1 has also been implicated as being neuroprotective in aging-related neurodegenerative disorders including Alzheimer's disease where Pin1 activity is diminished. Notably, recent proteomic analysis of brain samples from patients with mild cognitive impairment revealed that Pin1 is oxidized and also displays reduced activity. Since the Pin1 active site contains a functionally critical cysteine residue (Cys113) with a low predicted pK_a, we hypothesized that Cys113 is sensitive to oxidation. Consistent with this hypothesis, we observed that treatment of Pin1 with hydrogen peroxide results in a 32 Da mass increase, likely resulting from the oxidation of Cys113 to sulfinic acid (Cys-SO_2H). This modification results in loss of peptidyl-prolyl isomerase activity. Notably, Pin1 with Cys113 substituted by aspartic acid retains activity and is no longer sensitive to oxidation. Structural studies by X-ray crystallography revealed increased electron density surrounding Cys113 following hydrogen peroxide treatment. At lower concentrations of hydrogen peroxide, oxidative inhibition of Pin1 can be partially reversed by treatment with dithiothreitol, suggesting that oxidation could be a reversible modification with a regulatory role. We conclude that the loss of Pin1 activity upon oxidation results from oxidative modification of the Cys113 sulfhydryl to sulfenic (Cys-SOH) or sulfinic acid (Cys-SO_2H). Given the involvement of Pin1 in pathological processes related to neurodegenerative diseases and to cancer, these findings could have implications for the prevention or treatment of disease.

1. Introduction

Proline is unique among the amino acids in that the peptide bond found on the amino side of the proline can exist in both *cis* and *trans* configurations. Not surprisingly, the *cis–trans* isomerization of X-Pro bonds can have dramatic implications for the overall protein structure. In some cases, the *cis–trans* conversion occurs spontaneously, as a relatively slow event during protein folding [13]. The conversion can also be catalyzed by peptidyl-prolyl *cis–trans* isomerases that are members of three enzyme families: cyclophilins, FK506 binding proteins (FKBPs), and parvulins [11]. The former two families are well characterized as the target of the immunosuppressive drugs cyclosporine-A and FK506, respectively. The parvulin family includes Pin1, which is unique in that it is the only known phosphorylation-directed peptidyl-prolyl isomerase (K. P. [23,34,49]). Pin1 is comprised of two domains, namely an N-terminal WW domain and the catalytic peptidyl-prolyl isomerase (PPIase) domain. The WW domain binds to pSer/pThr–Pro motifs with high affinity, and is thought to play a role in substrate recruitment. The PPIase domain catalyzes the *cis–trans* interconversion of the pSer/pThr–Pro peptide bond.

Proline-directed phosphorylation has evolved as an important signaling mechanism in a variety of cellular processes (K. P. [25]). Given the ability of Pin1 to bind and isomerize pSer/pThr–Pro peptide bonds, it is envisaged that this enzyme plays a critical role in mediating protein conformational changes involved in these signaling events. The importance of Pin1 is evident from its involvement in human disease. Particularly relevant to this study, loss of Pin1 activity is implicated in neurodegeneration [7,36]. The extent of Ser/Thr–Pro phosphorylation increases in a number of neurodegenerative disorders including Alzheimer's disease (AD), where Tau structure and function are dramatically affected (K. P. [24]). Increased Pin1 expression reverses the deleterious properties of phosphorylated Tau (P.-J. [26,27,52]) and decreased Pin1 expression correlates with AD [21,40]. Consistent with a role in

Abbreviations: AD, Alzheimer's disease; Csd, cysteine sulfinic acid; DNPH, dinitrophenylhydrazine; DTDP, dithiodipyridine; MCI, 4,4′-mild cognitive impairment; PPIase, peptidyl-prolyl isomerase

* Corresponding author. Tel.: +1 519 661 4124; fax: +1 519 661 3175.
E-mail address: bshilton@uwo.ca (B.H. Shilton).
[1] Current affiliation: Department of Medical Biophysics, University of Toronto, Canada.

http://dx.doi.org/10.1016/j.bbadis.2014.12.025

maintenance of neuronal function, Pin1-null mice do not display developmental phenotypes but exhibit age-dependent behavioral and motor abnormalities, Tau filament formation, and signs of neuronal degeneration [21,30]. In addition, redox proteomic analysis of hippocampal samples from patients with either mild cognitive impairment (MCI) or AD shows increased levels of oxidative stress. Importantly, Pin1 was identified as a target of oxidation [4,12,43]. Interestingly, Pin1 also modulates the phosphorylation of neurofilament proteins in cortical neurons in response to oxidative stress [37] and has been implicated in neuronal apoptosis [2].

A key residue in catalysis by the PPIase domain is an active-site cysteine, Cys113, which appears to have an unusually low pK_a and is therefore ionized at cellular pH [3]. While substitution of Cys113 to alanine results in a complete loss of activity, considerable activity (approximately 30%) is retained when Cys113 is converted to aspartic acid. On this basis, and given its position in the active site, Cys113 may function to reduce the partial negative charge on the carbonyl oxygen of the substrate, and thereby reduce the peptidyl-prolyl double-bond character to facilitate *cis–trans* isomerization [3]. The low pK_a and catalytic importance of Cys113 are similar to the active site cysteine of protein tyrosine phosphatase PTP1b, which is regulated by oxidation [39]. In particular, oxidation of that residue by hydrogen peroxide results in the formation of a reversible sulphenyl-amide bond with the backbone amine of the following residue and consequent inhibition of phosphatase activity. More recently, redox regulation of the family of ovarian tumor (OTU) deubiquitinases has been identified [16]. These cysteine proteases are inhibited by hydrogen peroxide, which reversibly oxidizes the low pK_a catalytic cysteine to cysteine sulfenic acid. Analogous to PTP1b and the OTU deubiquitinase, we demonstrate in this work that wild-type Pin1, but not Pin1 harboring a Cys113Asp mutation, is sensitive to oxidation by hydrogen peroxide. This effect can be partially reversed by treatment with the reducing agent dithiothreitol (DTT). Using both biochemical and structural approaches we identify Cys113 as the site of oxidation. Taken together, these observations suggest that modulating the oxidation state of Pin1 could have important implications for the prevention of neurodegenerative disease.

2. Materials and methods

2.1. Protein expression and purification

The recombinant Pin1 used in biochemical and structural analyses contained a mutation of Arg14 to Ala, a substitution previously shown to enable crystallization [32]. A second derivative, $Pin1^{Cys113Asp}$, contained an additional mutation of Cys113 to Asp [3]. Both derivatives were expressed as hexa-histidine tagged fusions in *E. coli* strain BL21 from pProEX-HTA plasmids (Invitrogen). Single colonies were grown to an optical density of 0.6 at 600 nm in LB media at 37°, followed by overnight induction with 0.6 mM isopropylthio-α-D-β-galactoside at 18 °C. Bacteria were pelleted and resuspended in 50 mM sodium phosphate, 500 mM NaCl, 25 mM imidazole, pH 7.8 (buffer A) and the cell suspension frozen at −80°. Cells were thawed and supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/mL pepstatin A, and 10 μg/mL leupeptin) followed by lysis using a French press. The cell lysate was clarified by ultracentrifugation (100,000*g*) and applied to a 5 mL column of Ni-NTA Sepharose (GE Healthcare Life Sciences). The affinity resin was washed with 20 column volumes of buffer A and Pin1 was eluted using buffer A supplemented with 250 mM imidazole. To remove the affinity tag, TEV protease was added at a weight ratio of 1:25, along with 5 mM DTT, and the solution mixed gently at 20 °C for 2 hours; this was followed by a second addition of TEV protease and DTT. In preparation for cation-exchange chromatography, the solution was dialyzed against 5 mM sodium phosphate, 5 mM DTT, 1 mM EDTA, 20% glycerol, pH 7.0 (buffer B). The dialyzed Pin1 was applied to a 1.6 × 15 cm column of S-Sepharose HP (GE Healthcare) that was pre-equilibrated with buffer B, and eluted with a 300 mL gradient from 0 to 600 mM NaCl in buffer B. Fractions containing Pin1 were identified by SDS–PAGE, pooled, and concentrated by ultrafiltration to 15 to 20 mg/mL, and then dialyzed against 10 mM Hepes, 100 mM NaCl, 5 mM NaN_3, pH 7.4. Aliquots were flash frozen and stored at −80°.

2.2. Oxidation of Pin1

Hydrogen peroxide was added to a final concentration of either 500 μM or 1.0 mM to a 20 μM solution of Pin1. Incubation was continued as indicated (between 30 minutes and 4 hours) at 4° before performing subsequent assays or analysis. Where indicated, hydrogen peroxide was decomposed by adding 400 U of catalase (Sigma-Aldrich) at room temperature for 15 minutes, followed by incubation with 10 mM DTT for 30 minutes.

2.3. Peptidyl-prolyl isomerase assays

Peptidyl-prolyl isomerase assays were performed using Pin1 at a concentration of 0.5 μM. A 10 mM stock solution of the substrate was prepared by dissolving succinyl–Ala–Glu–Pro–Phe–*p*-nitroanaline in a water-free solution of 0.3 M LiCl in trifluoroethanol which maximizes the proportion of substrate with the Pro–Phe bond in a *cis* conformation. Chemical and Pin1-mediated *cis–trans* isomerase activity was measured by monitoring hydrolysis of the *trans* form of the substrate by chymotrypsin (50 mg/mL) as previously described [3,15].

2.4. Protein thiol determination assay

Determination of the thiol content of Pin1 was performed by reaction with 4,4′ dithiodipyrimidine (DTDP) using the protocol of Riener [35]. Absorbance readings were taken at 324 nm. For each experiment, background absorbance was determined with 120 μL of 20 μM Pin1 in Hepes buffer (50 mM Hepes, 150 mM NaCl, pH 7.0) followed by addition DTDP (5 μL of 4 mM DTDP in 12 mM HCl) with immediate mixing to yield a final concentration of 160 μM. After incubating for 50 minutes at 20 °C, the extent of the reaction was determined from the 4-thiopyridone by-product of the reaction, which absorbs light at 324 nm. The absorbance of a reagent control (A_{324r}), consisting of 120 μL of the Hepes buffer with 5 μL of 4 mM DTDP, was also measured. Solution was determined by the following equation:

$$[SH] = \frac{A_{324} - A_{324r}}{\varepsilon_{324} \times l},$$

where $\varepsilon_{324} = 21{,}400/\text{cm/M}$ and $l = 1$ cm.

2.5. Crystal structure analysis of peroxide-treated Pin1

Pin1 was crystallized by hanging drop vapor diffusion at 4 °C in 2.0 to 2.4 M ammonium sulfate, 1% (v/v) poly(ethylene glycol) 400 (PEG400) and 100 mM HEPES, pH 7.8. Oxidized crystals were grown as above, with mother liquor containing 10 mM hydrogen peroxide. Diffraction data were collected using X-rays at a wavelength of 1.5418 Å and processed with MOSFLM [18] and Scala [9]. The structure was determined by molecular replacement with starting model 2ITK [51]. Both molecular replacement and subsequent refinement were carried out with PHENIX [1], and Coot [8] was used for manual adjustment of the model. To calculate the isomorphous difference map in Fig. 4, data were collected from a Pin1 crystal grown under the conditions described above, but in the absence of peroxide (Table 1). To minimize differences due to scaling and merging, the two data sets (oxidized Pin1 and untreated native Pin1) were scaled together and then individually merged. These data sets were then used to calculate an isomorphous difference map ($|Fo_{ox}| - |Fo_{native}|$). The phases for the map were derived from a Pin1 model refined against the data from the oxidized crystal, but

Table 1
Crystallographic data and refinement.

	[a]Pin1 — untreated	[a]Pin1 — oxidized
Space group	$P3_121$	$P3_121$
Unit cell	$a = b = 68.66$, $c = 79.22$	$a = b = 68.53$, $c = 79.27$
Resolution	17.16-2.03 (2.14-2.03)	27.79–1.86 (1.96–1.86)
Rmerge	0.083 (0.554)	0.048 (0.190)
$I/\sigma I$	12.9 (2.5)	23.8 (7.5)
Completeness	94.5 (72.9)	98.4 (89.7)
Multiplicity	5.3 (4.6)	5.2 (4.8)
Refined model	[b]n/a	Residues 7 to 38 (WW domain) and 51 to 163 (PPIase); 1 PEG molecule; 4 sulfates; 201 waters
R/R_{free}		0.1528/0.1851 (0.2348/0.2661)
[c]Ramachandran (%)		
Most favored		94.4
Allowed		5.6
Generously allowed		0
Disallowed		0
RMSD		
Bond lengths (Å)		0.009
Bond angles (deg)		1.336
[d]Average B-factor		
Protein		24.8
Ligands		42.7
Solvent		35.7

[a] Values in parentheses refer to highest resolution shell.
[b] A molecular model was not refined against the untreated Pin1 data.
[c] Ramachandran statistics are from analysis with Procheck.
[d] B-factors were refined using 4 TLS groups, comprised of residues 7–62, 63–72, 73–98, and 99–163.

lacking any modification to Cys113. The refined structure of peroxide-treated Pin1 was deposited in the Protein Data Bank with ID 4QIB.

2.6. Electrospray ionization mass spectrometry

Pin1 samples were analyzed using a Q-TOF Ultima API (Waters, Milford, MA), with MassLynx V4.1 for analysis and data processing. Analysis parameters were as follows: capillary voltage, 3 kV; cone voltage, 60 V; RF lens 1 voltage, 40 V; source temperature, 80°; desolvation temperature, 250°. The cone and desolvation gas flow rates were 50 and 500 L/hour, respectively. The instrument was coupled to a Waters HPLC pump, using a water/acetonitrile gradient in the presence of 0.1% formic acid at 200 μL/min, and a BEH300 C4 (1.7 μm, 2.1 mm × 50 mm) reversed-phase column

2.7. Statistics

All statistics were computed in Prism6 (Graphpad Software), with *P* values determined by one-way ANOVA and Tukey's multiple comparison post-test using the indicated *n*-values. Clustering analysis of the isomerase rescue data (i.e. measurement of the isomerase activity of oxidized Pin1 following DTT treatment) was done using SciKit Learn, a Python-based machine learning library [31]. The *K*-means algorithm was initialized with three clusters, and fit to three factors: isomerase activity, hydrogen peroxide concentration, and duration of treatment.

3. Results

3.1. Pin1 is inhibited by oxidation

Cys113 of Pin1 is located within the enzyme's active site and is of prime importance for catalytic activity. This residue is thought to be ionized at physiological pH due to its low predicted pK_a [3]. This low pK_a in addition to other factors in the active site microenvironment could render the Cys113 thiol susceptible to attack by reactive oxygen species, including hydrogen peroxide [10,14], and oxidation of Cys113 would be expected to alter the activity of the enzyme. To assess the extent to which Pin1 activity is affected by oxidation *in vitro*, we purified recombinant Pin1 and treated it with hydrogen peroxide. The peptidyl-prolyl isomerase activity of purified Pin1 was measured using the peptide succinyl–Asp–Glu–Pro–Phe–*p*NA as substrate [3,15]. As shown in Fig. 1, the k_{cat}/K_M of Pin1 was 340 ± 60/mM/s, consistent with the previously determined value for recombinant Pin1 [3]. Oxidation of Pin1 was performed *in vitro* by incubating 20 μM Pin1 with 500 μM or 1.0 mM hydrogen peroxide, corresponding to molar ratios of 25:1 or 50:1 of hydrogen peroxide to Pin1, for 4 hours at 4 °C. The activity of oxidized Pin1 was decreased by both treatments, with the catalytic efficiency of oxidized Pin1 almost 100-fold lower than that of untreated Pin1 (Fig. 1).

To determine the extent and potential site of peroxide-mediated oxidation on Pin1, a cysteine-counting reagent, 4′4′-dithiodipyridine (DTDP), was used to monitor the loss of thiols [35]. Pin1 has two cysteine residues, Cys57 and Cys113, but only one reacts with DTDP (Fig. 2A). Pin1 with Cys113 substituted for aspartic acid (Pin1^{Cys113Asp}; [3]) showed zero reactive thiol groups per protein molecule, indicating that only Cys113 reacts with DTDP. Treating 20 μM Pin1 with either 500 μM or 1.0 mM hydrogen peroxide for 4 hours partially or fully eliminated the ability of Cys113 to react with DTDP, consistent with a peroxide-mediated oxidation of Cys113 (Fig. 2A). To confirm that the loss of Pin1 isomerase activity is due to oxidation of Cys113, the effect of oxidation on the isomerase activity of Pin1^{Cys113Asp} was measured. Treatment of Pin1^{Cys113Asp} with either 500 μM or 1 mM H_2O_2 for 4 hours had no significant effect on its catalytic activity (Fig. 2B). Taken together, these results strongly suggest that Cys113 is oxidized during H_2O_2 exposure, and this modification inhibits isomerase activity.

To assess the extent of Pin1 oxidation resulting from the *in vitro* hydrogen peroxide treatment, Pin1 was oxidized by exposure to 1.0 mM hydrogen peroxide for 4 hours and analyzed using mass spectrometry (Fig. 3A). Untreated Pin1 yielded a mass of 17583.2 Da, close to its theoretical value of 17582.6 Da, and this was increased by 32 Da after peroxide treatment, consistent with the addition of two oxygen atoms to Pin1. These findings are in agreement with recent top-down mass spectrometry experiments on hydrogen peroxide-treated Pin1, where Cys113 was oxidized to sulfinic acid (Cys-SO_2H) [42].

In general, cysteine can be oxidized to cysteine sulfenic acid (Cys-SOH) or to sulfinic acid (Cys-SO_2H). Oxidation to cysteine sulfenic acid can be reversed by reducing agents such as DTT, whereas oxidation to cysteine sulfinic acid is irreversible [5]. To determine whether modification to sulfenic acid or sulfinic acid is the cause of Pin1 inhibition, we

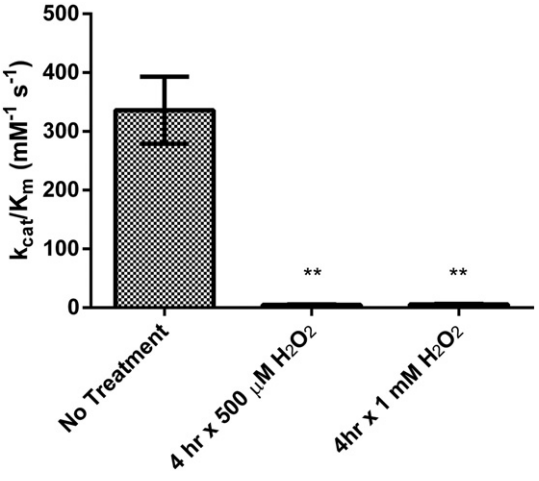

Fig. 1. Pin1 is inhibited by hydrogen peroxide. Pin1 isomerase activity was determined by a spectrophotometric assay using the peptide substrate succinyl–Ala–Glu–Pro–Phe–*p*-nitroanaline [3]. Pin1 at a concentration of 20 μM was incubated with 500 μM or 1.0 mM hydrogen peroxide for 4 hours prior to the assay. $^{**}P \leq 0.01$, $n = 3$.

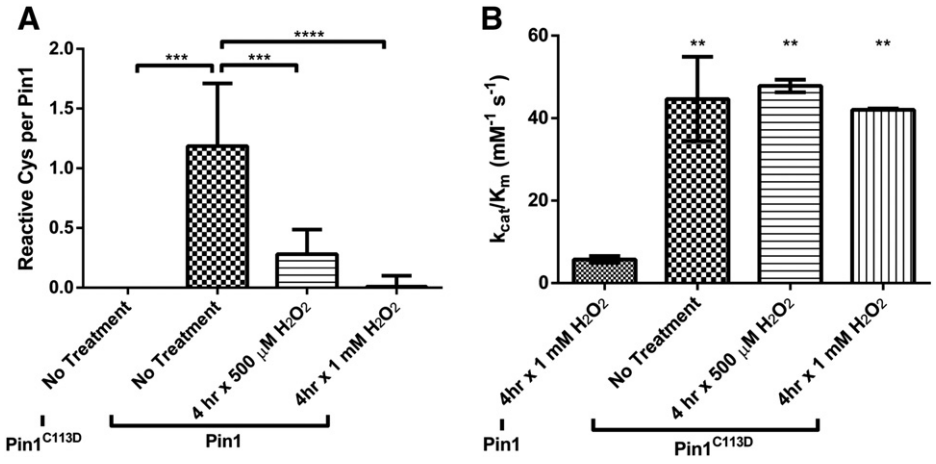

Fig. 2. Peroxide treatment of Pin1 leads to loss of the Cys113 thiol. (A) The effect of oxidation on the number of reactive thiol groups was assessed with 4,4′-dithiodipyridine (DTDP; [35]). A 20 μM solution of Pin1 or Pin1^{Cys113Asp} was pre-incubated with 500 μM or 1.0 mM hydrogen peroxide for 4 hours followed by incubation with DTDP for 5 minutes at 20 °C. Absorbance was measured at 324 nm, and after subtracting background controls, the concentration of thiopyridine-modified cysteine was determined from the amount of 4-thiopyridone released during the reaction. $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$, $n = 6$. (B) The isomerase activity of Pin1^{Cys113Asp} was measured using succinyl–Ala–Glu–Pro–Phe–*p*-nitroanaline as substrate after being treated with hydrogen peroxide at concentrations of 500 μM or 1.0 mM for 4 hours. Note that activity of Pin1^{Cys113Asp} is typically 30% of the activity of wild-type Pin1 [3]. $^{**}P \leq 0.01$, $n = 3$.

assessed the ability of reducing agents to reverse the peroxide-mediated loss in catalytic activity. Pin1 (20 μM) was incubated with 1.0 mM hydrogen peroxide for 4 hours at 4° and then catalase was added to remove excess hydrogen peroxide. Treatment of the oxidized Pin1 with DTT (10 mM over 30 minutes) recovered only a very small amount of activity (Fig. 3B). Taken together, these results indicate that treatment of Pin1 with 1 mM hydrogen peroxide over 4 hours leads to almost complete conversion of Cys113 to sulfinic acid, with consequent loss of catalytic activity.

3.2. Peroxide treatment of Pin1 specifically converts Cys113 to cysteine sulfinic acid

X-ray crystallography was used to positively identify Cys113 as the site of modification, and to assess structural changes attendant upon peroxide treatment of Pin1. Crystals of Pin1 were grown in the presence of 10 mM peroxide and after 3 days crystallographic data were collected (Table 1). The structure was solved using native Pin1 (PDB ID: 2ITK; [51]) as a starting model. Refinement proceeded essentially to completion (R/R_{free} values of 0.1536/0.1848) without including peroxide-mediated modifications to any side chains. At this point, the phases from the model were used to calculate an isomorphous difference map ($|Fo_{ox}| - |Fo_{native}|$) between data collected from a Pin1 crystal grown in the presence of 10 mM peroxide, and data from another crystal grown under identical conditions but in the absence of peroxide (Table 1). The largest peaks in the resulting $|Fo_{ox}| - |Fo_{native}|$ electron density map were connected with Cys113, and when the $|Fo_{ox}| - |Fo_{native}|$ map was contoured at 5σ, the only electron density present was associated with Cys113 (Fig. 4A). Additional Pin1 residues that might be prone to peroxide oxidation are Met15, Met130, Met146, and Cys57; of these, only Met15 showed a small peak of positive electron density when the $|Fo_{ox}| - |Fo_{native}|$ map was contoured at 3σ.

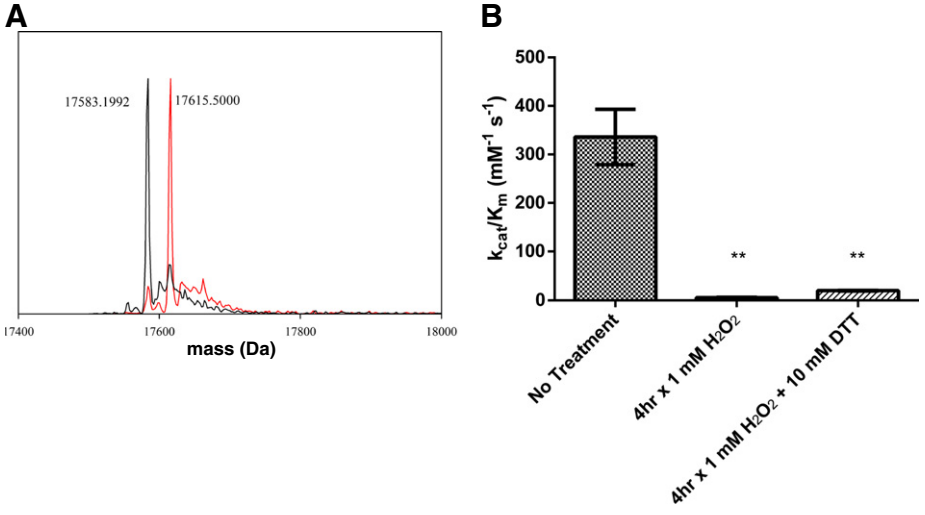

Fig. 3. Peroxide-mediated oxidation of Pin1 results in 32 Da mass shift accompanying loss of activity. (A) The effect of peroxide treatment on the molecular mass of Pin1. Deconvoluted ESI mass spectra of untreated Pin1 (black trace) and Pin1 treated with 1.0 mM hydrogen peroxide for 4 hours (red trace) are shown. The Pin1 spectrum shows one major peak at 17583 Da; upon oxidation, this peak shifts 32 Da to 17615 Da, indicating the addition of two oxygen atoms to Pin1. (B) Effect of peroxide treatment on Pin1 isomerase activity. Pin1 that had been subjected to the peroxide treatment leading to a 32 Da mass shift was tested for isomerase activity to assess the degree of reversibility. A 20 μM solution of Pin1 was incubated with 1.0 mM hydrogen peroxide for 4 hours, and then quenched by adding 400 U of catalase for 15 minutes, followed by 10 mM DTT for 30 minutes. Treatment with DTT did not significantly increase activity ($P > 0.5$), consistent with conversion of a thiol to sulfinic (Cys-SO_2H) rather than sulfenic (Cys-SOH) acid. $^{**}P \leq 0.01$, $n = 3$.

Met15 is highly solvent exposed and in the WW domain, and so any oxidation of this residue is unlikely to affect the PPIase activity of Pin1. Thus, differences in crystallographic data from crystals grown in the presence and absence of peroxide indicate that Cys113 is the site of oxidation.

The identity of Cys113 as the site of oxidation was further supported by a difference density map, |Fo| − |Fc|, derived using the observed amplitudes from the oxidized Pin1 crystal, along with amplitudes and phases calculated from the refined Pin1 structure outlined above. In this case, the two highest electron density peaks in the map (11.8σ and 9.5σ) were 1.5 Å from the γ-S atom of Cys113 (Fig. 4B); these peaks represent electron density that is contributing to the diffraction, but is not present in the partially refined model. There was no significant electron density associated with any other sulfur atoms in the structure, even when contoured at relatively low level of 2σ. The positions of the two electron density peaks observed in the |Fo| − |Fc| map were fully consistent with the pyramidal geometry expected for cysteine sulfinic acid, and therefore structure refinement was completed with a cysteine sulfinic acid residue (Csd instead of Cys) at position 113. The final structure and electron density map (2|Fo| − |Fc|) are shown in Fig. 4C. There are only small changes in the overall structure of oxidized Pin1, with an average distance between α-carbon (CA) positions of 0.526 Å. Therefore, peroxide-mediated oxidation of Pin1 specifically converts Cys113 to cysteine sulfinic acid, and produces only a local structural change in the protein. For the crystallographic studies, the treatment with hydrogen peroxide was relatively harsh (10 mM H_2O_2 over several days) compared to that used to produce catalytically inactive Pin1 (1 mM H_2O_2 over several hours). On this basis, the crystallographic analysis indicates that Cys113 is uniquely susceptible to oxidation (since no other residues were modified under these conditions), but that oxidation of Cys113 does not proceed to the fully oxidized sulfonic acid (Cys-SO_3H; [48]).

Oxidation of Pin1 is partially reversible. Peroxide-mediated oxidation proceeds first with the formation of sulfenic acid (Cys SOH) that can be followed by oxidation to sulfinic acid (Cys-SO_2H). We were curious as to whether the less stable sulfenic acid form could be observed by milder treatment with hydrogen peroxide, which would open the possibility that Pin1 isomerase activity is reversibly regulated by physiological redox mechanisms [5]. Pin1 was treated with either 500 μM or 1 mM peroxide for various times, after which the reaction was stopped by adding catalase to consume any additional peroxide. Isomerase assays were performed on samples after oxidation or following an additional treatment for 30 minutes with 10 mM DTT (Fig. 5A). The least harsh oxidation conditions (500 μM peroxide for 30 minutes) resulted in 85% loss of activity that could be partially reversed by DTT treatment. More extensive exposure to hydrogen peroxide resulted in almost complete inhibition of Pin1 activity, but with 30 to 40% recovered after the DTT treatment. These results show that a significant proportion of Pin1 is oxidized only to the sulfenic acid (Cys SOH) form under mild oxidizing conditions, and that this form, like the sulfinic acid form, has a much lower catalytic activity than native Pin1.

From the graph in Fig. 5A, it appears as though the efficacy of rescue dropped in a stepwise rather than linear manner as concentration and duration of peroxide treatment was increased. To assess whether there are discrete differences in the response of Pin1 to varying treatments of hydrogen peroxide, rather than a continuous linear trend,

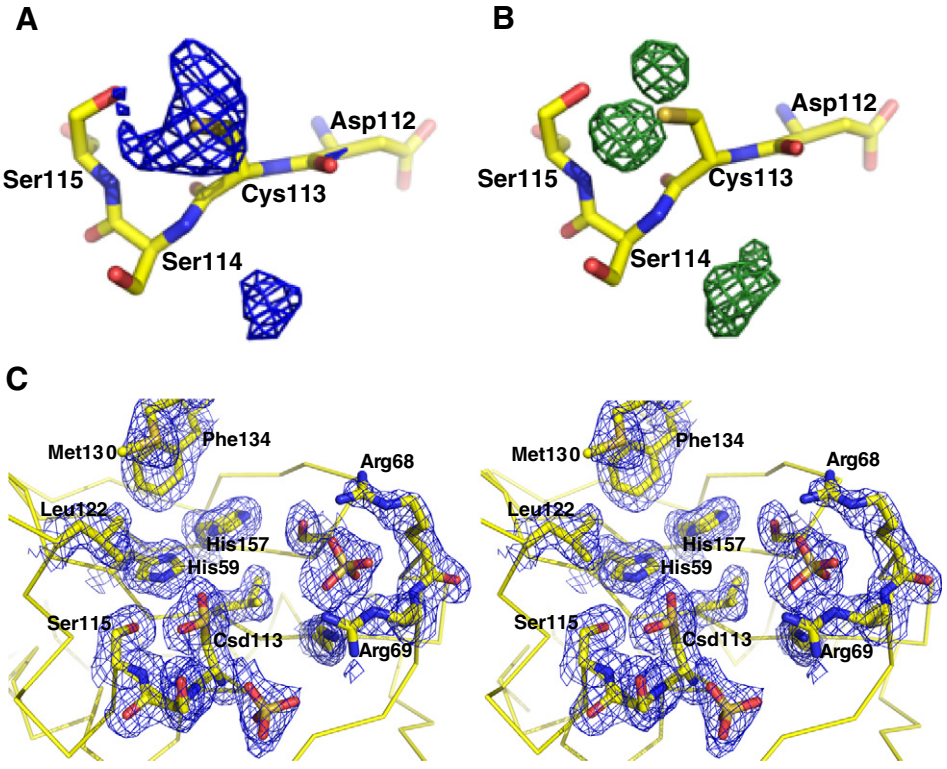

Fig. 4. Peroxide-mediated structural changes in Pin1. In panels A and B, difference electron density maps show the effect of peroxide treatment on the structure of Pin1. In both cases, the maps indicate an increase in electron density associated with the γ-sulfur of Cys113 for crystals grown in the presence of peroxide. (A) An isomorphous difference electron density map ($|Fo_{oxidized}| - |Fo_{native}|$, blue mesh, contoured at 5σ) was calculated from differences in crystallographic data collected from Pin1 crystals grown in either the presence or absence of 10 mM peroxide. Phases were derived from the partially refined Pin1 structure. (B) A difference electron density map (|Fo| − |Fc|, green mesh, contoured at 5σ) was derived from the difference between crystallographic data collected from Pin1 crystals grown in the presence of peroxide, and calculated amplitudes and phases from the partially refined Pin1 structure. (C) A stereo view of the active site of peroxide-treated Pin1, in which Cys113 has been converted to cysteine sulfinic acid (Csd113) on the basis of the difference electron density maps in panels A and B. The electron density map (2|Fo| − |Fc|) is derived from the final refined structure of peroxide-treated Pin1, which was deposited in the Protein Data Bank with ID 4QID. Images were created using the PyMOL Molecular Graphics System, Version 1.6.0.0 Schrödinger, LLC.

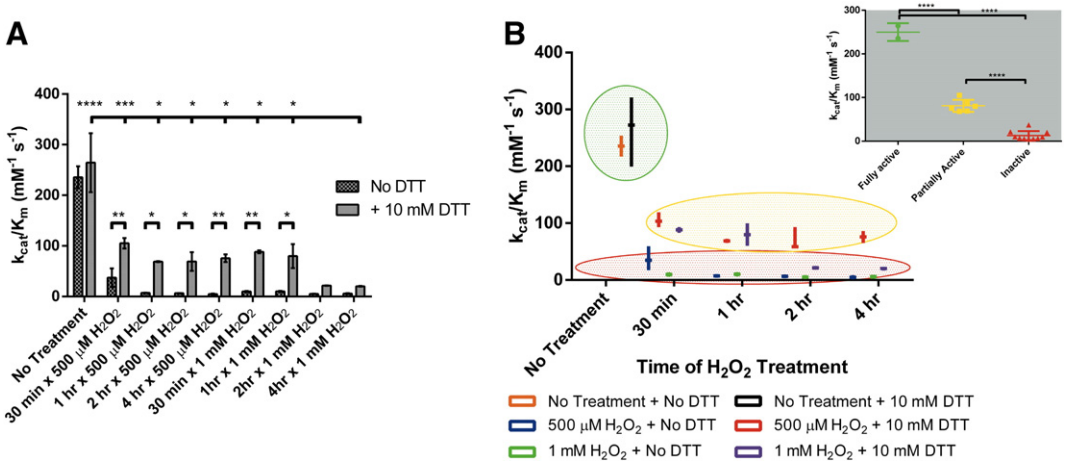

Fig. 5. Inhibition of Pin1 by oxidation can be partially reversed. (A) Isomerase activity of Pin1 after oxidation and DTT treatment is shown. A 20 μM solution of Pin1 was incubated with 500 μM or 1.0 mM hydrogen peroxide for up to 4 hours, and then quenched by adding 400 U of catalase for 15 minutes, followed by 10 mM DTT for 30 minutes. Effect of H_2O_2 and DTT treatments were measured by ordinary two-way ANOVA, corrected for multiple comparison by the Holm–Sidak method. Effect of DTT was significant at $^{**}P \le 0.01$ and $^{*}P \le 0.05$ for intermediate H_2O_2 treatments. Simple effects of H_2O_2 treatments + DTT were significant at $^{*}P \le 0.05$, $^{***}P \le 0.001$, and $^{****}P \le 0.0001$ ($n = 3$ for each group) when compared to the most extreme treatment (4 hours × 1 mM). Similarly, comparing the effect of H_2O_2 treatments to the no treatment positive control was significant at $P \le 0.0001$ for all treatments (not shown). (B) *K*-means clustering analysis of the above data. Samples were plotted as dot and whiskers representing mean and range respectively. Each cluster is circled in the color in which it appears in the inset. The inset illustrates that the clusters identified had significantly different isomerase activity. $^{****}P \le 0.0001$.

K-means clustering analysis was used (Fig. 5B). *K*-means clustering of the data from Fig. 5A was carried out using three factors: isomerase activity, peroxide concentration, and duration of treatment. This analysis separated the data into three groups: fully active, partially active (approximately 30% activity), and inactive. The untreated samples were both classified as fully active, while all samples treated with hydrogen peroxide, but not DTT, were classified as inactive. All samples treated with both peroxide and DTT, except those treated with 1.0 mM peroxide for 2 or 4 hours, clustered together as partially active, meaning that their activities were nearer to each other than the activities of the fully active or inactive samples. In order for the isomerase activity of oxidized Pin1 to be rescued by DTT-mediated reduction, Cys113 must have been modified to the less stable sulfenic acid (Cys SOH) rather than sulfinic acid (Cys-SO_2H). The fact that 30–45% of isomerase activity is rescued over a range of treatments suggests that a distinct and persistent subset of Pin1 molecules are able to maintain the unstable sulfenic acid moiety at Cys113, while the remaining molecules are further oxidized to sulfinic acid. At this point, a mechanism by which the sulfenic acid form of Cys113 may be stabilized in Pin1 is not known.

4. Discussion

4.1. Pin1 is inhibited by oxidation of the catalytic Cys113

The current investigation was initiated because two oxidative proteomics studies of brain tissue from individuals with mild cognitive impairment (MCI) or Alzheimer's disease (AD) demonstrated that Pin1 is significantly oxidized, and Pin1 activity reduced, compared to tissue from individuals not suffering from MCI or AD [4,43]. The exact nature of the oxidation was not clear, since the reagent used to detect oxidized proteins, dinitrophenylhydrazine (DNPH), reacts with aldehydes, ketones, and also cysteine sulfenic acid [6]. We have extended this work to show that Pin1 activity is lost upon treatment with hydrogen peroxide *in vitro*, and determined the site of oxidation to be the catalytic cysteine, Cys113. Hydrogen bonding in the Pin1 active site is predicted to lower the pK_a of Cys113 to approximately 6.5 [19], which would increase the concentration of the reactive thiolate anion at neutral pH; however, as with other redox-sensitive active site cysteines, there must be additional factors in the Pin1 active site that make Cys113 unusually susceptible to oxidation [10].

With gentle peroxide treatment, Cys113 undergoes partial oxidation to the reversible sulfenic acid. Thus, *in vitro*, the active site cysteine of Pin1, Cys113, is sensitive to oxidation, can be reversibly oxidized to cysteine sulfenic acid, and is irreversibly oxidized to cysteine sulfinic acid. The redox proteomics studies of the hippocampus of patients with MCI and AD identified oxidized proteins based on the presence of peptide carbonyl derivatives that would react with DNPH [4,43,44]. The generation of peptide carbonyl derivatives, or carbonylation, is generally the result of metal-catalyzed oxidation or severe oxidation and can occur at most amino acids [33]. Our *in vitro* studies show that Cys113 is by far the most redox sensitive residue in Pin1, and, since oxidation of cysteine to sulfenic or sulfinic acid requires less oxidative stress than the generation of peptide carbonyl derivatives, it follows that Pin1 identified as carbonylated in hippocampal samples of patients with MCI and AD would contain Pin1 that is oxidized at Cys113. In this regard, it should be noted that the reagent used to detect the oxidative modifications in brain tissues (DNPH) does not react with cysteine sulfinic acid [6], and so the actual form detected was most likely restricted to Cys113 reversibly oxidized to the sulfenic acid. In summary, combining the results of the current study with the previous oxidative proteomics studies [4,43], oxidation of Pin1 *in vivo* is most likely occurring on Cys113 to yield cysteine sulfenic acid, with possibly some irreversible oxidation to sulfinic acid, and both modifications result in loss of Pin1 activity.

Why is a redox-sensitive cysteine present in the active site of Pin1? A partial or full negative charge on Cys113 is thought to contribute to the catalytic mechanism [3]. Consistent with this idea is the fact that Cys113 can be substituted with aspartic acid, which is found in the active site of related parvulins [41], and $Pin1^{Cys113Asp}$ retains 25 to 30% of its activity and fully supports viability in yeast [3]. $Pin1^{Cys113Asp}$ is not only functional, but also resistant to oxidation, which raises the question as to whether cysteine in the active site has been selected for because it allows Pin1 to be modulated by oxidative signaling mechanisms. Pin1 is a phospho-specific peptidyl-prolyl isomerase and as such has been implicated in kinase-mediated signaling pathways [38,50]. In fact, a number of both phosphatases and kinases have redox-sensitive cysteine residues that modulate enzyme activity when oxidized [5]. The presence of a redox-sensitive cysteine in the active site of Pin1 therefore raises the possibility that it is normally subject to redox regulation in cells.

4.2. The relationship between oxidation of Pin1 and neurodegenerative pathologies

Given its potential roles in kinase-mediated signaling, it is expected that Pin1 will have diverse roles in a number of cellular processes. Pin1 knockout mice display accelerated neurodegeneration and a variety of other symptoms associated with premature aging [17,21,22]. Given that Pin1 could be neuroprotective (K. P. [25]), an effort has been made to understand why it does not effectively perform that role in neurodegenerative diseases. While there is little evidence to suggest that there is a genetic link, or that Pin1 expression changes in AD, Pin1 availability and subcellular localization is negatively affected in AD pathologies [27–29,40,47]. Pin1 associates with both neurofibrillary tangles and amyloid plaques in neurons from AD patients [36]. This causes a reduction in the soluble fraction of Pin1, and a change in localization from the nucleus to cytoplasm ([21]; P.-J. [26,45,46]). Since Pin1 prevents or reverses both of these pathologies, there may be a relationship between the loss of Pin1 activity and AD [20,30]. These studies suggest a role for Pin1 in the progression of AD with the neuroprotective functions of Pin1 being lost as age-related oxidative stress inhibits Pin1, presumably allowing the formation of neurofibrillary tangles and β-amyloid deposits. In turn this could further promote the loss of Pin1 function by sequestering it to the insoluble fraction and thereby enhancing the disease pathology.

Overall, this work has revealed that Pin1, a phosphorylation-dependent peptidyl-prolyl isomerase, undergoes oxidation at Cys113, a residue with a critical role in catalysis. Given the apparent involvement of Pin1 in pathological processes related to neurological disorders, this finding could have important implications for the development of strategies for the prevention or treatment of neurodegenerative diseases. Furthermore, in light of its functions related to the control of cell division, it is conceivable that oxidation of Pin1 could be exploited for therapeutic intervention in proliferative disorders such as cancer.

Acknowledgements

We would like to thank Melanie Bailey for constructing several of the Pin1 derivatives. This work was supported by an Operating Grant from the Cancer Research Society Inc. to D.L., L.K., C.B. and B.S. B.I. was supported by a WGRS studentship from the University of Western Ontario.

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