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Oxidatively induced Cu for Mn exchange in protein phosphatase 1γ: A new method for active site analysis

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ABSTRACT

Protein phosphatase 1γ, a serine/threonine phosphatase, is a metalloprotein that coordinates two Mn^{2+} in the active site when expressed in *Escherichia coli* in a buffer containing $MnCl_2$. Herein, we report on the oxidatively induced copper for manganese exchange in protein phosphatase 1γ, thus enabling firm confirmation of the four histidine (His) amino acid residues (His66, His125, His173, and His248) involved in metal coordination. By exchanging manganese with copper the oxidation yields for the peptides increased dramatically, thus simplifying detection of the oxidized peptides and analysis of the oxidation sites within the oxidized peptides. We also found that when copper was added during the oxidation process a new metal coordination center was formed at cysteine 39, 105, 140, and 155.

1. Introduction

Currently as many as one-half of all proteins are metalloproteins and the majority of these proteins contain transition metals as part of their structure.[1] Protein phosphatase 1 (PP1), which is a serine/threonine phosphatase,[2] belongs to this group of proteins. PP1 is found in all tissues and is involved in a range of cellular processes such as cell cycle progression, protein synthesis, muscle contraction, carbohydrate metabolism, transcription, neuronal signaling, learning and memory.[2a–c] Expressed in *Escherichia coli* in a buffer containing $MnCl_2$ PP1 contains two Mn^{2+} in the active site.[3] Although it has not been unambiguously proved, it is thought that natural PP1 contains Fe^{2+} and Zn^{2+} in the active site based on its sequential similarities with protein phosphatase 2B (calcineurin) (see Fig. S1 in the electronic Supplementary data).[4]

Amino acid residues in the vicinity of the metal center(s) in copper containing metalloproteins can be selectively oxidized by addition of H_2O_2, which upon reaction with Cu^+ generates reactive oxygen species (ROS) via a Fenton-like reaction (a general outline of this reaction is shown in Fig. 1).[5] This has been successfully demonstrated for Cu,Zn-superoxide dismutase (Cu,Zn-SOD) protein by our group[6] and other groups[7] using mass spectrometry for post oxidation analysis. Although it has been known for some time that

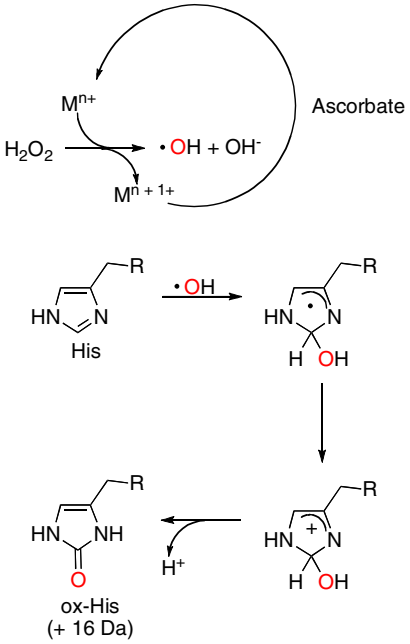

Figure 1. General outline of the Fenton/Fenton-like reaction (top) and an example of how the reactive oxygen species generated from that reaction oxidizes histidine.

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first-row transition metal ions other than Cu (i.e., Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+}) under appropriate reaction conditions can generate ROS,[8] and for this work the ability of Mn^{2+} to form ROS is of particular interest.[9] However, it was Vachet and co-workers who demonstrated that these metal ions also are capable of selectively oxidizing amino acid residues engaged in metal coordination.[10] This was showcased in two metal coordinating peptides, where for example the metal coordinating histidines (His6 and His9) in angiotensin I were selectively oxidized upon exposure to H_2O_2 and ascorbate or $S_2O_8^{2-}$ and ascorbate. Although the oxidation yields were variable when Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+} was used, and in most cases much lower than for Cu^{2+} the method seemed to bear some promise for analysis of the metal binding site in non-Cu-binding metalloproteins.[10] To the best of our knowledge there is no report, apart from a comment in Vachet's paper where they refer to promising preliminary work on Fe-SOD,[10] where this strategy has been extended to analysis of metalloproteins containing Mn^{2+}, Fe^{2+}, Co^{2+}, or Ni^{2+} in the active site.

Our group have for some time been interested in studying metallopeptides and metalloproteins,[11] and lately we[6,12] and others[13] have been involved in research aiming at elucidating the metal coordination sites of metalloproteins using oxidation by ROS generated by for example treatment with H_2O_2 and sodium ascorbate, followed by MS analysis. Another long term research project within the group is to elucidate the binding site of tautomycin with PP1γ using various strategies.[14] As a part of this work, we herein report on the oxidatively induced metal exchange (Mn^{2+}→Cu^{+}/Cu^{2+}) strategy for the oxidation labeling of PP1γ's active site. By using this strategy all four His involved in metal coordination in PP1γ could be detected.

2. Results and discussion

2.1. Background oxidation

Upon treatment of PP1γ with H_2O_2 (5 mM) and sodium ascorbate (10 mM) in buffer, peptides **T9**, and **T15** were oxidized (+16 Da) in 3% yield (Table 1), as evident from nano-LC-ESI-Q-TOF-MS of the trypsin digested protein. Further analysis of these peptides in order to determine the point of oxidation utilizing nano-LC-ESI-IT-MS/MS only resulted in poor and inconclusive data due to the low yield of the oxidized peptides. Firm confirmation of the oxidation site in the two peptides was therefore difficult due to the fact that these two peptides also contain potentially readily oxidized cysteine (Cys) amino acid residues (Fig. 2 and Fig. S2 in the electronic Supplementary data). However, based on literature precedent, which states that Cys-SOH is unstable and easily undergoes further oxidation to Cys-SO_2H (+32 Da) and Cys-SO_3H (+48 Da) or reacts with thiol [dithiothreitol (DTT) was used during digestion of the protein; see experimental part for details] to yield disulfide,[15] it is most likely that the site of oxidation in the two peptides is His66 for peptide **T9** and His125 for peptide **T15**. Apart from these two peptides, no substantial oxidation was detected besides the already determined background oxidation [Cys127 in **T15**, methionine 183 (Met183) in **T22**, Met316 in **T34**, as well as one oxidized amino acid residue in peptides **T24** and **T31** (most likely Cys or Met)].

Table 1
Oxidation yields for peptides **T9**, **T15**, **T22**, and **T29** at various concentrations of added Cu^{2+} ($CuSO_4$)[a]

Peptide	0 equiv Cu^{2+} (%)[b]	0.5 equiv Cu^{2+} (2.85 μM) (%)	1.0 equiv Cu^{2+} (5.70 μM) (%)	3.0 equiv Cu^{2+} (17.0 μM) (%)
T9 + 16 Da	3	20	34	38
T15 + 16 Da	3	18	24	32
T22 + 16 Da	0	10	11	21
T29 + 16 Da[c]	0	10	14	27

[a] Oxidation yields are calculated as indicated in note 16.
[b] Background oxidation.
[c] The oxidation yield for this peptide was estimated since one other peptide overlaps with this peptide in the LC chromatogram.

2.2. Oxidatively induced Cu^{+}/Cu^{2+} for Mn^{2+} exchange

When Cu^{2+} ($CuSO_4$) and sodium ascorbate (ascorbate reduces Cu^{2+} to Cu^{+})[17] was added during the oxidation process several additional oxidations took place as detected by nano-LC-ESI-Q-TOF-MS analysis of the peptides derived from trypsin digestion of the protein post oxidation. This finding was intriguing and optimization experiments were conducted in order to find the best oxidation conditions. The outcome of these experiments were monitored by nano-LC-ESI-Q-TOF-MS by analyzing the whole oxidized protein as well as analysis of the peptides derived from tryptic digestion of the oxidized protein. By such means we found the optimum concentration of oxidant to be 5 mM H_2O_2 in combination with 10 mM sodium ascorbate as reducing agent (see experimental part for details). The optimum conditions were decided based on the oxidation yields obtained for the various peptides.

With the optimum concentration of H_2O_2 and sodium ascorbate established we conducted a range of experiments with various concentrations of copper added during the oxidation. By using nano-LC-ESI-Q-TOF-MS we found that the oxidation yields for peptides **T9**, **T15**, **T22** and **T29** gradually rose when the concentration of copper increased (0.5→3.0 equiv) (Table 1). Further analysis of these peptides with nano-LC-ESI-IT-MS/MS enabled us to confirm that His involved in metal coordination was oxidized selectively in peptides **T9** (His66) and **T15** (His125) as evident from the MS/MS spectra depicted in Figures 3 and 4, respectively.

Under conditions including copper the last two peptides, viz. **T22**, and **T29** containing a His amino acid residue (His173, and His248, respectively) engaged in metal coordination could also be oxidized in a maximum of 21% and 27%, respectively, when 3 equiv of Cu^{2+} was employed (Table 1). MS/MS analysis of peptide **T22** only enabled us to elucidate that the site of oxidation is situated somewhere on one of the following amino acid residues; isoluecine 169 (Ile169), phenylalanine 170 (Phe170), Cys171, Cys172, His173, glycine 174 (Gly174), or Gly175 (Fig. 5). Ile, and Gly can be ruled out as oxidation sites due to the fact that they are not likely to be oxidized under these conditions.[5] Cys is easily oxidized by ROS and can therefore not automatically be ruled out as the site of oxidation. However, based on the arguments used previously regarding the oxidation of Cys amino acid residues[16] (vide supra) we could also rule out Cys171, and Cys172 as the oxidation site due to the fact that the mass increase for peptide **T22** was only 16 Da, thus leaving Phe170 or His173 as the potential oxidation site. However, based on our previous experience it is most likely that His173 is the point of oxidation.[6,12] An assumption that is confirmed by the already reported X-ray crystal structure of PP1, which shows that Phe170 is remote from the metal center (>10 Å from the metal center).[3]

Further analysis of peptide **T29** with nano-LC-ESI-IT-MS/MS showed that the amino acid residue that had been oxidized was either alanine 247 (Ala247), His248 or glutamine 249 (Gln249) (see Fig. 6). From this data we can conclude that the oxidation site is His248 since the two other amino acid residues are not likely to be oxidized under the conditions used in this work.[5]

The initial oxidation, under conditions excluding copper, points towards ROS being generated upon reaction between H_2O_2 and Mn^{2+}. However, the oxidation yields were quite low (**T9** 3% and **T15** 3%), and for two of the four His containing peptides involved in metal binding the oxidized peptide could not be detected. The addition of copper greatly increased the yield of the oxidized

1 7 6 21 27 37 44
MADLDK LNIDSIIQR LLEVR GSKPGK NVQLQENEIR GLCLK SR EIFLSQPILLELEAPLK
T1 T2 T3 T4 T5 T6 T7 T8

61 75 99 114
ICGDIHGQYYDLLR LFEYGGFPPESNYLFLGDYVDR GK QSLETICLLLAYK IK YPENFFLLR
T9 T10 T11 T12 T13 T14

123 133 144 151
GNHECASINR IYGFYDECK R R YNIK LWK TFTDCFNCLPIAAIVDEK
T15 T16 T17 T18 T19 T20 T21

169 189 212 222
IFCCHGGLSPDLQSMEQIR R IMRPTDVPDQGLLCDLLWSDPDK DVLGWGENDR GVSFTFGAEVVAK
T22 T23 T24 T25 T26

235 239 247 262
FLHK HDLDLICR AHQVVEDGYEFFAK R QLVTLFSAPNYCGEFDNAGAMMSVDETLMCSFQILKPAEK
T27 T28 T29 T30 T31

303 315 320
K KPNATRPVTPPR GMITK QAK K
T32 T33 T34 T35 T36

Figure 2. Peptide map for trypsin digested PP1γ. His engaged in metal coordination is marked in red (peptide **T9**, **T15**, **T22**, and **T29**).

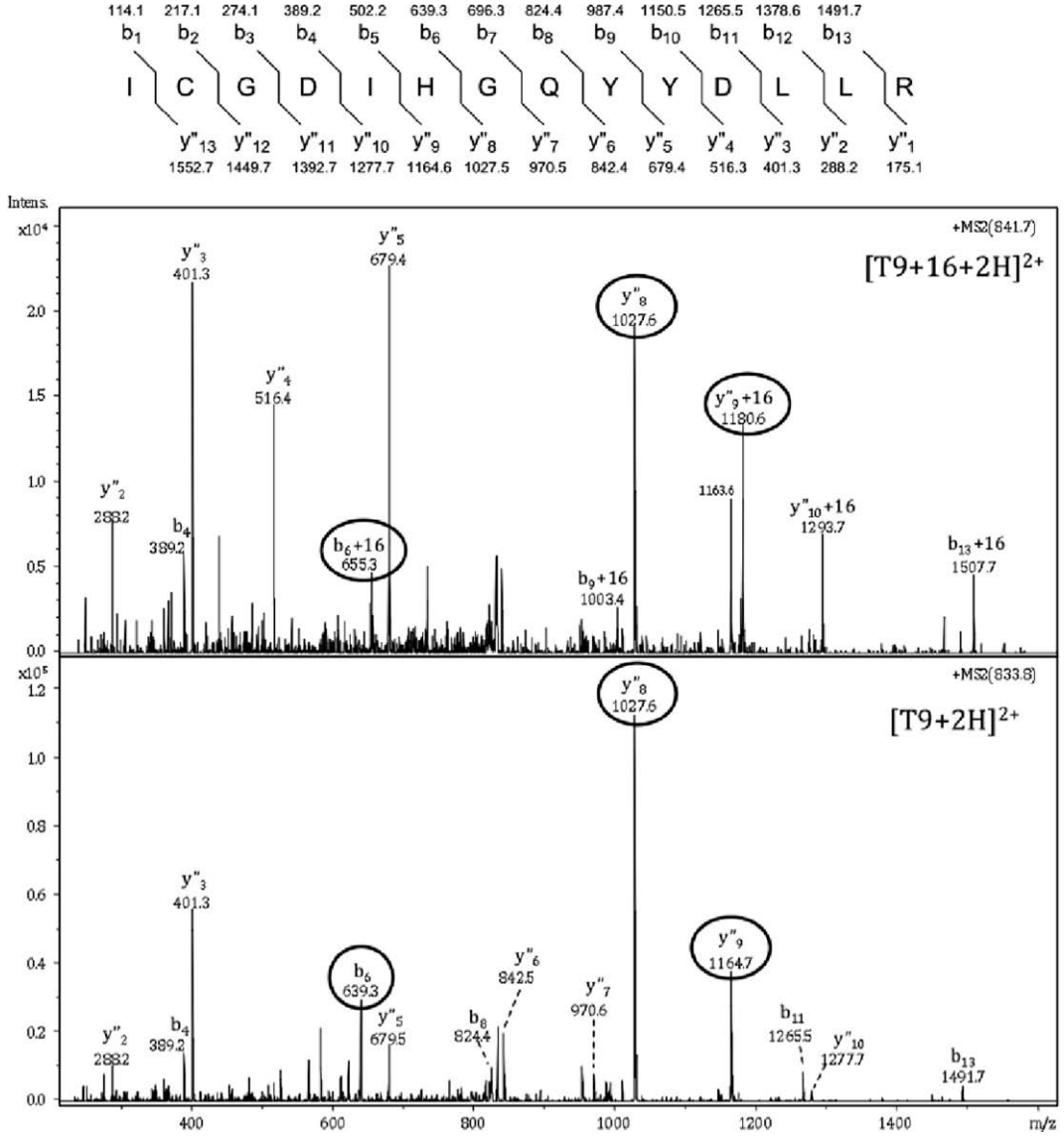

Figure 3. MS/MS spectra of peptides **T9** and **T9** + 16 Da. Peptide **T9** + 16 Da shows a mass increase of 16 Da for fragments y''_9 and y''_{10} compared with peptide **T9**. The spectrum of peptide **T9** + 16 Da also shows that fragment y''_8 is unchanged compared with peptide **T9**, thus confirming the oxidation of His66. The oxidation of His66 is also supported by a 16 Da increase in the mass of fragment b_6 in the spectrum of peptide **T9** + 16 Da compared with peptide **T9**.

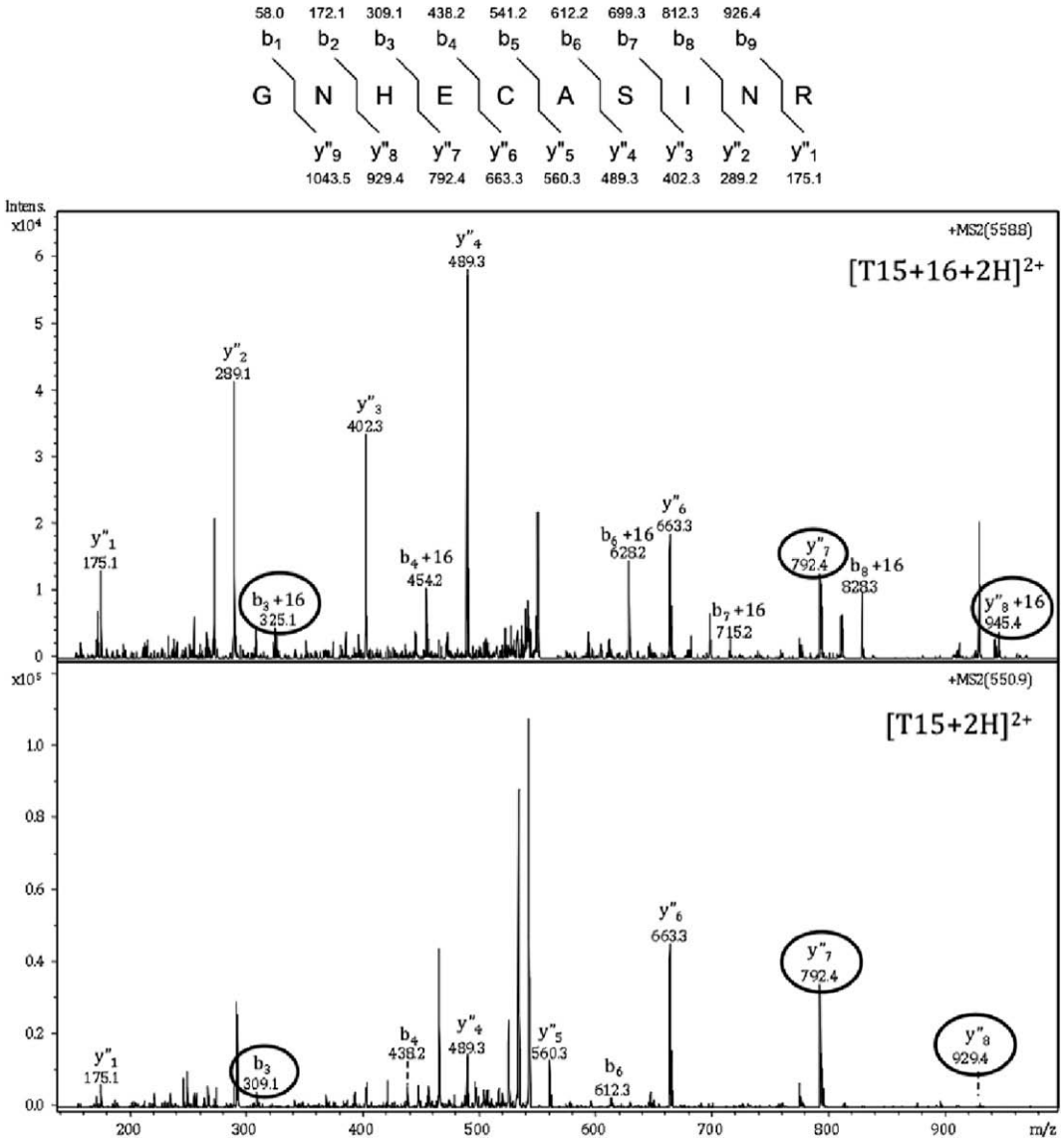

Figure 4. MS/MS spectra of peptides **T15** and **T15** + 16 Da. Peptide **T15** + 16 Da shows a mass increase of 16 Da for fragment y''_8 compared with peptide **T15**. The spectrum of peptide **T15** + 16 Da also shows that fragment y''_7 is unchanged compared with peptide **T15**, thus confirming the oxidation of His125. The oxidation of His125 is also supported by a 16 Da increase in the mass of fragment b_3 in peptide **T15** + 16 Da compared with peptide **T15**.

peptides, thus making it possible to confirm the His amino acid residues involved in metal coordination in PP1γ (Fig. 7).

2.3. Plausible mechanism for metal exchange

We postulate that the increased oxidation yields after addition of copper is due to exchange of metal in the active site of the protein from Mn^{2+}/Mn^{3+} to Cu^{+}/Cu^{2+}. Currently we have no direct evidence as to how the metal exchange takes place in the active site of PP1γ, however, it is not so likely that the metal is exchanged prior to oxidation (vide infra). Our current working hypothesis is that Mn^{2+} may rapidly be oxidized to Mn^{3+} by H_2O_2 and in the process generating ROS. Mn^{3+} in the active site is then exchanged with Cu^{+}/Cu^{2+}. With Cu^{+} in place of Mn^{2+} the generation of ROS becomes much more efficient and it is possible to improve the oxidation yields for the amino acid residues around the metal centers, thus making it easier to be detect by LC–MS analysis after tryptic digestion.

In efforts aiming to prove the oxidation state of manganese at the stage of metal exchange we conducted activity measurements of PP1γ with *para*-nitrophenyl phosphate with various concentrations of $CuSO_4$ in the solution. These tests showed that even when large excess of Cu^{2+} (up to 1 mM, i.e., 175 equiv) added to the reaction mixture had practically no suppressive effect on the activity of PP1γ toward *para*-nitrophenyl phosphate. From work by Lee and co-workers[4e] we know that PP1 containing Cu^{2+} in the metal center has no activity. Therefore, the fact that PP1γ keeps its activity in the presence of Cu^{2+} indicates that no metal exchange is taking place when manganese is in oxidation state 2+. This finding indirectly supports our working hypothesis that the metal exchange first takes place after Mn^{2+} has been oxidized to Mn^{3+}. This result also shows that the higher structure of PP1γ remains unchanged even in a solution with high excess of copper ions.

The method reported herein might be possible to implement for the analysis of other metalloproteins containing first-row transition metals such as Mn^{2+}, Fe^{2+}, Co^{2+}, and Ni^{2+}, however, we have not yet tested this methodology on proteins other than PP1γ. Therefore comments regarding the generality of this method has to await further investigations.

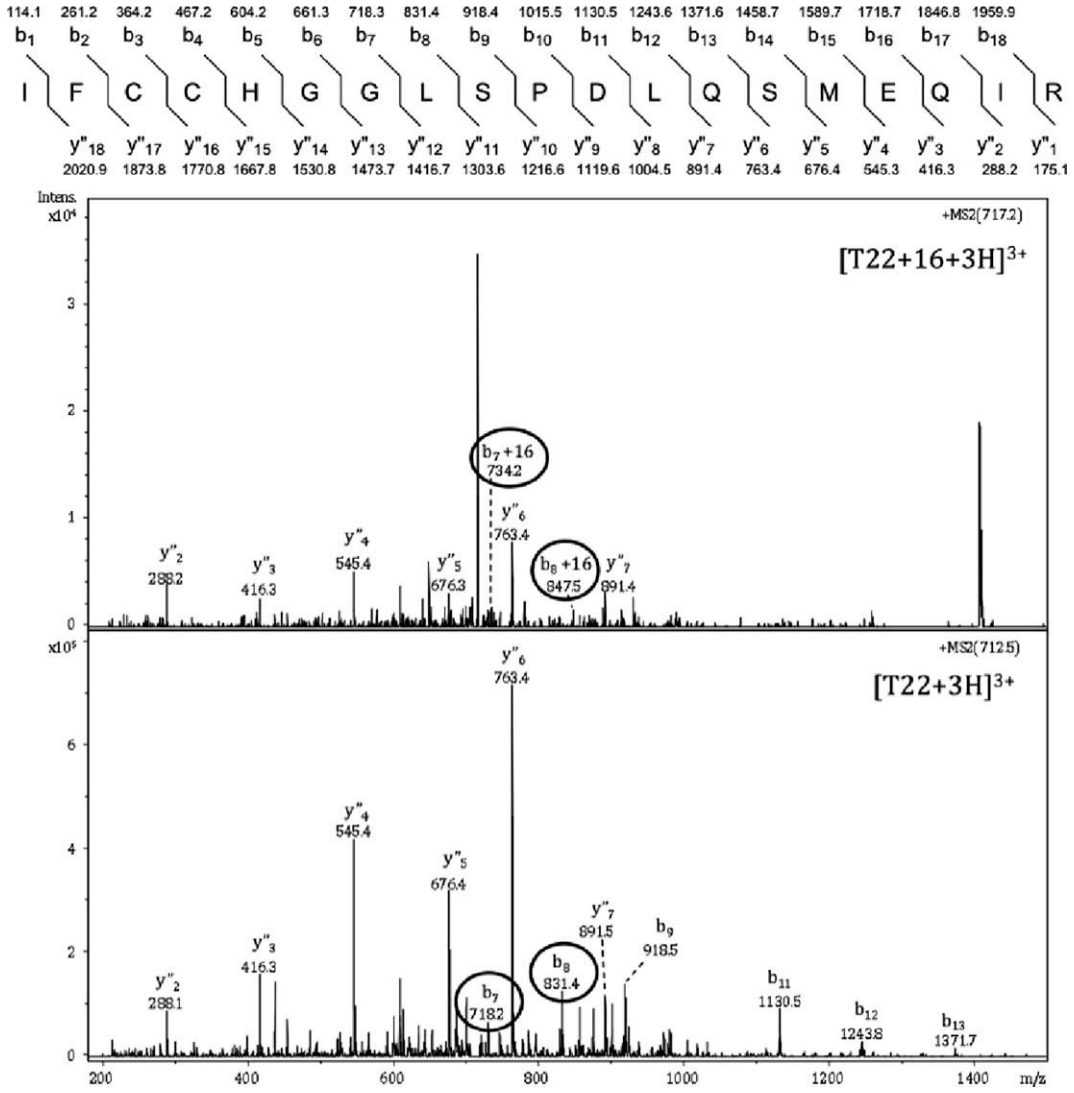

Figure 5. MS/MS spectra of peptides **T22** and **T22** + 16 Da. Peptide **T22** + 16 Da shows a mass increase of 16 Da for fragments b_7 and b_8 compared with peptide **T22**, thus indicating that the oxidation has taken place at one of the following amino acid residues; Ile169, Phe170, Cys171, Cys172, His173, Gly174 or Gly175.

In previous experiments with bovine Cu,Zn-SOD, we found similar metal exchange taking place, although under different conditions (first treatment with EDTA, then addition of metal ions).[18] In this case, Cu and Zn ions were displaced by $2Cu^{2+}$, $2Zn^{2+}$, $2Ag^{+}$, and $2Co^{2+}$.

2.4. Additional oxidations under conditions including Cu^{+}/Cu^{2+}

Addition of Cu^{2+} during the oxidation reaction also resulted in successful oxidation of other peptides, which were not found to be oxidized when Cu^{2+} was not added to the reaction mixture, as evident from extensive nano-LC-ESI-Q-TOF-MS analysis (Table 2). Peptides **T6** (+32 Da, and +48 Da), **T10** (+16 Da), **T12** (+32 Da), **T16** (+32 Da), and **T21** (+32 Da) predominantly increased in mass by 32 Da except for peptide **T10**, thus indicating that the Cys amino acid residue within peptides **T6**, **T12**, **T16**, and **T21** were predominantly oxidized. This assumption was confirmed by conducting further MS and MS/MS analysis on the respective peptides, which clearly showed that Cys in peptide **T6** was oxidized. The MS/MS spectrum of peptide **T21** revealed that the site of oxidation was among one of the following amino acid residues; threonine 151 (Thr151), Phe 152, Thr153, aspartic acid 154 (Asp154) or Cys155. However, a mass increase of 32 Da indicates that Cys155 is the only amino acid residue that has been modified. Despite numerous efforts, it was not possible to obtain good quality MS/MS data for peptides **T12** and **T16**. However, the mass increase of 32 Da points towards Cys amino acid residue within these two peptides being the point of oxidation. Since these peptides are not oxidized under experimental conditions meant to unravel background oxidation or under conditions excluding copper, it is clear that these peptides are oxidized due to the addition of copper. It seems likely that apart from Cu^{+}/Cu^{2+} exchanging with Mn^{2+} in the active site that also copper is coordinated in a newly generated metal center. It is well known that Cys amino acid residues that are not involved in disulfide bonds can coordinate metals in metalloproteins, such as for example in metallothioein **1a**.[19] We therefore postulate that the Cys amino acid residues in peptides **T6**, **T12**, **T16**, and **T21** coordinates Cu^{2+} in a new metal center, and that ROS generated around this metal facilitates the oxidation of the coordinating Cys amino acid residues (Fig. 8).[20] The MS/MS analysis of peptide **T21** also confirmed that only one of the two Cys amino acid residues was oxidized in this peptide, namely Cys155. Cys158, which is also included in peptide **T21** (see Fig. 2), remained unoxidized most likely due to the fact that Cys158 is facing away from the new metal center (see Fig. S3 in the electronic Supplementary data).

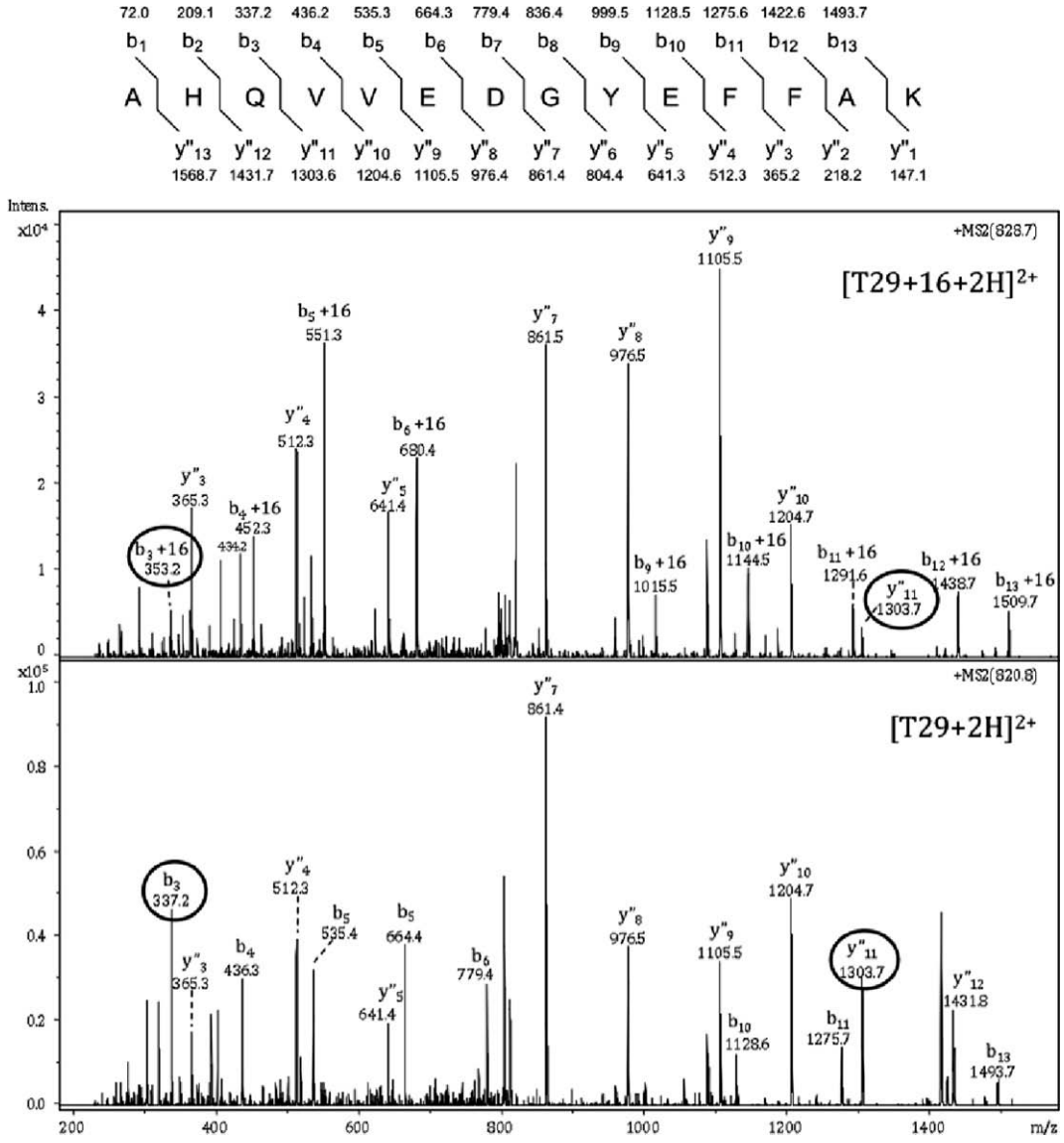

Figure 6. MS/MS spectra of peptide **T29** and **T29** + 16 Da. Peptide **T29** + 16 Da shows a mass increase of 16 Da for fragment b_3 compared with peptide **T29**. The spectrum of peptide **T29** + 16 Da also shows y''_{11} unchanged compared with peptide **T29**, thus confirming that the oxidation has taken place at one of the following amino acid residues; Ala247, His248 or Gln249.

MS/MS analysis of peptide **T10**, which does not contain any Cys amino acid residue (see Fig. 2), only revealed that the site of oxidation were either leucine 75 (Leu75), Phe76, glutamic acid 77 (Glu77), tyrosine 78 (Tyr78), Gly79, Gly80, Phe81, proline 82 (Pro82), Pro83, Glu84, serine 85 (Ser85), aspartic acid 86 (Asn86), Tyr87, or Leu88. However, based on our previous experience where we found that Pro can be oxidized under these conditions[6] and the fact that amino acid residues Leu, Glu, Gly, Ser, and Asn are not readily oxidized under the conditions used in this work,[5] we concluded that the point of oxidation is most likely at one of the following amino acid residues; Phe76, Tyr78, Phe81, Pro82, Pro83, or Tyr87 (Fig. 9). The oxidation of peptide **T10** seems to be nonspecific due to its remote distance from any of the metal centers, but reproducible. Attempts to reduce the amount of oxidation of peptide **T10** by for example increasing the amount of sodium ascorbate, a method previously used with success by Vachet and coworkers,[7d] were not successful.

3. Conclusion

Although the oxidation yields for the amino acid residues involved in metal binding in PP1γ are rather modest under conditions where ROS is generated by Mn^{2+} it is still possible to elucidate two out of four His amino acid residues involved in metal coordination, thus proving that metals other than copper can be utilized for this type of analysis in metalloproteins. By adding Cu^{2+} and sodium ascorbate during the oxidation we have developed a new supplementary method for analysis of the active site of PP1γ by exchanging Mn^{2+} with Cu^{+}/Cu^{2+}. The generality of this method is still unproved, however, if this method is found to be general it opens up a new alternative method for metal site analysis in metalloproteins containing metals other than copper. The metal exchange is carried out by adding $CuSO_4$ to the solution of PP1γ prior to addition of sodium ascorbate and H_2O_2. Under conditions including copper a new metal center was generated by Cys39, Cys105, Cys140, and Cys155

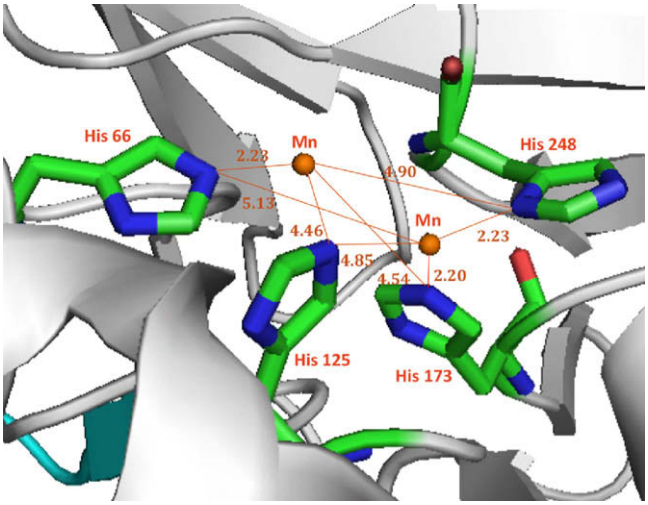

Figure 7. The active site of PP1 with the two metals marked in orange. The distance from the two metals to His is indicated in Å in the figure. The figure is based on the X-ray structure (3e7b) obtained from the protein data bank.[1]

Table 2
Oxidation yields for peptides **T6**, **T10**, **T12**, **T16**, and **T21** at various concentrations of Cu^{2+}[a]

Peptide	0 equiv Cu^{2+} (%)[b]	0.5 equiv Cu^{2+} (2.85 μM) (%)	1.0 equiv Cu^{2+} (5.70 μM) (%)	3.0 equiv Cu^{2+} (17.0 μM) (%)
T6 + 32 Da	0	4	5	6
T6 + 48 Da	0	3	4	4
T10 + 16 Da	0	7	9	11
T12 + 32 Da	0	0	35	45
T16 + 32 Da	0	7	11	13
T21 + 32 Da	0	8	9	17

[a] Oxidation yields are calculated as indicated in note 16.
[b] Background oxidation.

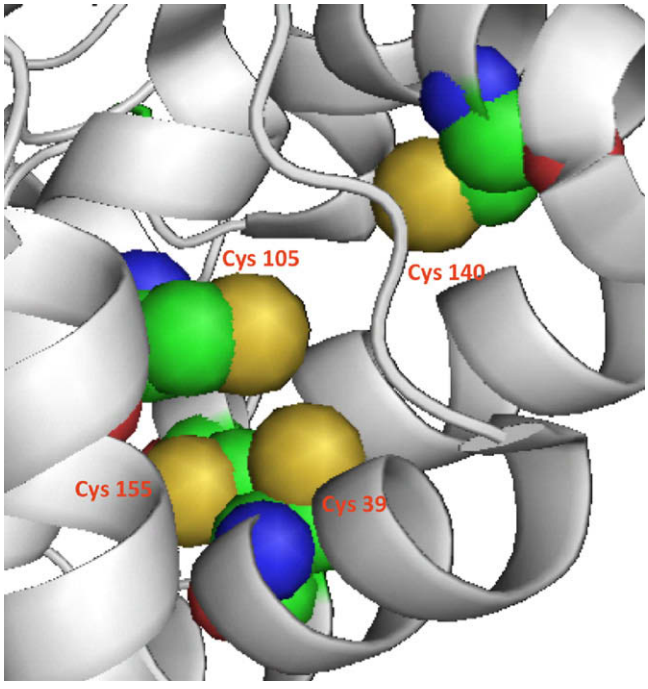

Figure 8. New metal center in PP1γ formed upon addition of Cu^{2+}: Cys involved in Cu^{2+} coordination are highlighted (Cys39, Cys105, Cys140, and Cys155). The figure is based on the X-ray structure (3e7b) obtained from the protein data bank.[3b]

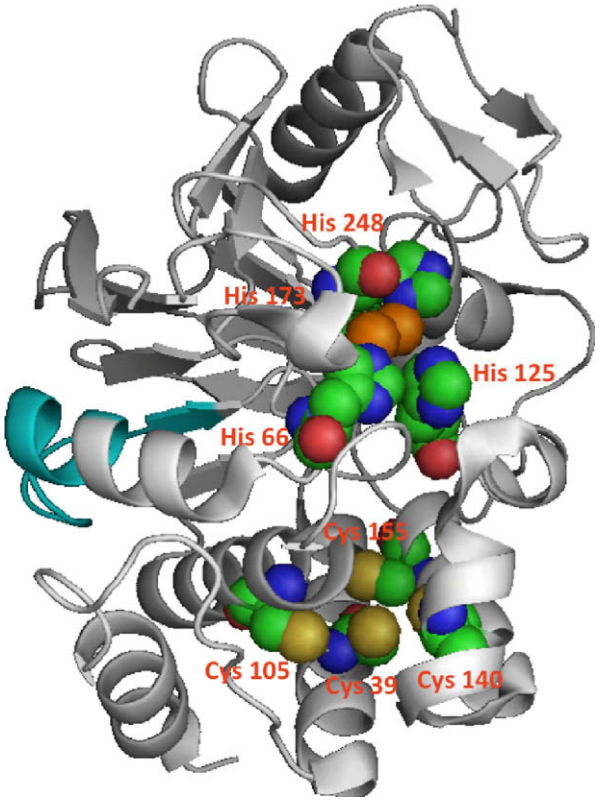

Figure 9. The X-ray structure of PP1 with the active site highlighted (His66, His125, His173, and His248), the new metal center highlighted (Cys39, Cys105, Cys140, and Cys155), and the potential oxidation sites on peptide **T10** marked in light blue. The figure is based on the X-ray structure (3e7b) obtained from the protein data bank.[3b]

coordinating to Cu^{+}/Cu^{2+}, thus resulting in selective oxidation of the coordinating Cys amino acid residues.

4. Experimental section

4.1. Instrumentation

MS spectra were recorded utilizing a Q-TOF mass spectrometer (Micromass, Manchester, UK) equipped with a Z-spray type ESI source. Data were acquired and processed using MassLynx version 4.0. All samples were desalted and separated by non-split type pre-packed gradient (PPG) system and appropriately adjusted nano-HPLC system (JASCO, Tokyo, Japan) using a Develosil ODS-HG-5 column (Nomura, 150 mm × 0.3 mm i. d.) before on-line ESI-MS analysis. The column was equilibrated with 260 μL water containing 0.025% trifluoroacetic acid at a flow rate of 10 μL/min and then developed using a linear gradient from 0% to 100% of acetonitrile containing 0.025% trifluoroacetic acid for 40 min at a flow rate of 5 μL/min. The column effluent was monitored at 210 nm and then introduced into the electrospray nebulizer without splitting.

MS/MS spectra were recorded utilizing an ion trap (IT) HCT Plus mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an orthogonal ESI source. Data were acquired and processed using Compass version 1.0 (esquireControl™ and DataAnalysis™ version 3.2) (Bruker Daltonics), respectively. All MS experiments were preformed in the positive ion mode.

4.2. Chemicals

H_2O_2 (30%) and dithiothreitol (DTT) was purchased from Nacalai Tesque (Kyoto, Japan); sodium ascorbate was purchased from TCI (Tokyo, Japan). Trypsin (sequence grade) was purchased from

Roche Diagnostics (Mannheim, Germany). Acetonitrile (HPLC grade) for nano-LC-ESI-Q-TOF-MS was purchased from Nacalai Tesque (Kyoto, Japan) and acetonitrile (HPLC grade) for nano-LC-ESI-IT-MS/MS was purchased from Roche (Mannheim, Germany).

5. Experimental

5.1. Preparation and purification of PP1γ

A full-length cDNA encoding γ-isoform of PP1 (PP1γ) was expressed in *E. coli* (provided by Dr. Patricia Cohen, Dundee, U.K.), as described previously.[21] The expressed PP1γ was purified by sequential chromatography at 4 °C on five columns: HiPrep Q-XL, HiPrep SP-XL, phenyl-Sepharose, Sephacryl S-200 and Mono-Q (all purchased from Amersham-Pharmacia Biotech, Uppsala, Sweden). Buffers used for the expression and purification of PP1γ contained $MnCl_2$ (1 mM) Two-dimensional electrophoresis [isoelectric focusing on an IPG gel (pH 3–10 L type; Amersham-Pharmacia) rehydrated in urea (8 M), DTT (20 mM), ampholyte (0.5%) and CHAPS (2%), followed by SDS/PAGE] of the purified PP1γ revealed a single polypeptide spot at pI 6.0 and apparent molecular mass 37 kDa. The molar concentration of PP1γ was determined, as described previously,[22] by a titration procedure using microcystin-LR (provided by Dr. Ken-ichi Harada, Meijo University, Nagoya, Japan) as the standard.

5.2. Activity assay

Assay of phosphatase activity were carried out at 25 °C essentially by the procedure described previously.[21] Briefly, *p*NPP phosphatase activity was measured by monitoring the increase in the absorbance at 405 nm resulting from accumulation of the reaction product *p*-nitrophenol, using a spectrophotometer connected to a pen-recorder. The buffer used contained: Tris (base) (40 mM), $MgCl_2$ (34 mM), EDTA (free acid) (4 mM) and DL-dithiothreitol (4 mM). No divalent cation other than Mg^{2+} was added. The pH of this solution (without adjustment) was 8.4, which is optimal for the *p*NPP phosphatase activities of PP1. The specific activity for PP1γ was 0.29 ± 0.03 μmol-Pi liberated/min/mg protein ($n = 64$), when it was measured at 15 °C using *p*NPP (5.0 mM) as the substrate.

5.3. Procedure for determining background oxidation

To a stock solution of PP1γ (15 μL of a 17.2 μM solution, 258 pmol) was added tris–HCl buffer (16.45 μL), and the resulting solution was incubated at 25 °C for 30 min. H_2O (6.45 μL) was then added to the mixture followed by incubation at 25 °C for 30 min. H_2O (5 μL) was then added to the mixture and the resulting mixture was incubated at 25 °C for 30 min. H_2O_2 (2.5 μL of a 100 mM solution) was added to the mixture and the resulting solution was incubated at 37 °C for 30 min. The reaction mixture was then diluted by addition of water (50 μL) and the resulting solution was frozen and freeze-dried. In order to remove trace of H_2O_2 additional water (50 μL) was added and the sample was frozen and freeze-dried (repeated twice). The sample obtained by such means was diluted with water (6.7 μL) and subjected to tryptic digestion.

5.4. General procedure for oxidation of PP1γ (Mn^{2+})

To a stock solution of PP1γ (15 μL of a 17.2 μM solution, 258 pmol) was added tris–HCl buffer (16.45 μL), and the resulting solution was incubated at 25 °C for 30 min. H_2O (6.45 μL) was then added to the mixture followed by incubation at 25 °C for 30 min. Sodium ascorbate (5 μL of a 100 mM aq solution) was then added to the mixture and the resulting mixture was incubated at 25 °C for 30 min. H_2O_2 (2.5 μL of a 100 mM solution) was added to the mixture and the resulting solution was incubated at 37 °C for 30 min. The reaction mixture was then diluted by addition of water (50 μL) and the resulting solution was frozen and freeze-dried. In order to remove trace of H_2O_2 additional water (50 μL) was added and the sample was frozen and freeze-dried (repeated twice). The sample obtained by such means was diluted with water (6.7 μL) and subjected to tryptic digestion.

5.5. General procedure for oxidation of PP1γ with copper (1 equiv)

To a stock solution of PP1γ (15 μL of a 17.2 μM solution, 258 pmol) was added tris–HCl buffer (16.45 μL), and the resulting solution was incubated at 25 °C for 30 min. $CuSO_4{\cdot}5H_2O$ (6.45 μL of a 40 μM aq solution, 258 pmol) was then added to the mixture followed by incubation at 25 °C for 30 min. Sodium ascorbate (5 μL of a 100 mM aq solution) was then added to the mixture and the resulting mixture was incubated at 25 °C for 30 min. H_2O_2 (2.5 μL of a 100 mM solution) was added to the mixture and the resulting solution was incubated at 37 °C for 30 min. The reaction was then quenched by addition of water (50 μL) and the resulting solution was frozen and freeze-dried. In order to remove trace of H_2O_2 additional water (50 μL) was added and the sample was frozen and freeze-dried (repeated twice). The sample obtained by such means was diluted with water (6.7 μL) and subjected to tryptic digestion.

5.6. Tryptic digestion of PP1γ or oxidized PP1γ

The aq. solution of PP1γ or oxidized PP1γ was heated at ca. 90 °C for 5 min in order to denature the protein. The sample was then cooled to room temperature and a solution of trypsin [4.8 μL, of a 0.1 μg/μL in tris–HCl buffer, (5% w/w of PP1γ)] was then added and the resulting solution was incubated at 37 °C for 20 h. Trypsin was then deactivated by heating the solution at ca. 90 °C for 5 min. After cooling the sample to room temperature, a solution of DTT (1 μL of a 120 mM aq solution) was added and the solution was incubated at 37 °C for 2 h. The resulting sample was stored at −20 °C until it was subjected to nano-LC-ESI-Q-TOF-MS or -MS/MS analysis.

5.7. Preparation of tris (hydroxymethyl) aminomethan-HCl (tris–HCl) buffer

Tris-(hydroxylmethyl)aminomethane (121.0 mg, 1.0 mmol), NaCl (117.0 mg, 2.0 mmol) and DTT (46.0 mg, 0.30 mmol) were dissolved in distilled water (90 mL) and pH was then adjusted to 8.43 (at 25 °C) by addition of 1 M HCl (aq solution). The volume was then adjusted to 100 mL by adding distilled water.

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Supplementary data

Figures S1–S3 and MS/MS spectra of peptides **T6**, **T10**, and **T21** are included as supplementary materials. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.10.014.

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16. Calculation of oxidation yields are performed using the following function, where ***TX*** = signal intensity in the ion chromatogram:

$$\mathbf{T}X + 16\,\mathrm{Da} = \frac{\mathbf{T}X + 16\,\mathrm{Da}}{\mathbf{T}X + (\mathbf{T}X + 16\,\mathrm{Da}) + (\mathbf{T}X + 32\,\mathrm{Da}) + (\mathbf{T}X + 48\,\mathrm{Da}) \times 100}.$$

17. Ascorbate reduces Cu^{2+} to Cu^{+}. Cu^{+} reacts with H_2O_2 and generates ROS via a Fenton-like reaction (see Ref. 5 for literature regarding Fenton and Fenton-like reactions) and Cu^{2+}. When excess of ascorbate is used, which is the case in the work described herein, Cu^{+} is regenerated upon reaction with another equivalent of ascorbate.
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