RESEARCH NOTE

Evidence for the oxidant-mediated amino acid conversion, a naturally occurring protein engineering process, in human cells [version 1; referees: 1 approved, 2 not approved]

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Abstract

Reactive oxygen species (ROS) play an important role in the development of various pathological conditions as well as aging. ROS oxidize DNA, proteins, lipids, and small molecules. Carbonylation is one mode of protein oxidation that occurs in response to the iron-catalyzed, hydrogen peroxide-dependent oxidation of amino acid side chains. Although carbonylated proteins are generally believed to be eliminated through proteasome-dependent degradation, we previously discovered the protein de-carbonylation mechanism, in which the formed carbonyl groups are chemically eliminated without proteins being degraded. Major amino acid residues that are susceptible to carbonylation include proline and arginine, both of which are oxidized to become glutamyl semialdehyde, which contains a carbonyl group. The further oxidation of glutamyl semialdehyde produces glutamic acid. Thus, we hypothesize that through the ROS-mediated formation of glutamyl semialdehyde, the proline, arginine, and glutamic acid residues within the protein structure are interchangeable. In support of this hypothesis, mass spectrometry demonstrated that proline 45 (a well-conserved residue within the catalytic sequence) of the peroxiredoxin 6 molecule can be converted into glutamic acid in cultured human cells, establishing a revolutionizing concept that biological oxidation elicits the naturally occurring protein engineering process.

Open Peer Review

Referee Status: ✔ ✘ ✘

	Invited Referees 1	2	3
version 1 published 28 Apr 2017	✔ report	✘ report	✘ report

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Michael J. Davies , University of Sydney, Australia

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Comments (0)

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Introduction

Reactive oxygen species (ROS) are produced through the electron reduction of molecular oxygen and include superoxide anion radicals, hydrogen peroxide (H_2O_2), and hydroxyl radicals (Freeman & Crapo, 1982; Halliwell & Gutteridge, 2007). ROS have been implicated in the pathogenesis of various diseases (Freeman & Crapo, 1982; Halliwell & Gutteridge, 2007), as well as in the aging process (Harman, 1956). One electron reduction of molecular oxygen produces superoxide, which in turn reacts with each other to produce H_2O_2 and reduces cellular iron ions. Reduced iron donates an electron to H_2O_2 and produces highly reactive hydroxyl radicals. Hydroxyl radicals in turn react with virtually all biological molecules, including DNA, proteins, lipids and small molecules, damaging the biological system (Freeman & Crapo, 1982; Halliwell & Gutteridge, 2007).

One important event that occurs in response to the metal (iron)-catalyzed oxidation process is the formation of carbonyls in the protein structure. Protein carbonylation has been shown to be increased in various diseases and in aging (Berlett & Stadtman, 1997; Levine & Stadtman, 2001; Levine, 2002; Stadtman *et al.*, 1988). Protein carbonylation occurs in response to the iron-catalyzed, H_2O_2-dependent oxidation of amino acid side chains (Stadtman, 1990; Suzuki *et al.*, 2010). Protein carbonylation inactivates protein functions and marks damaged proteins for proteasome-dependent degradation (Grune *et al.*, 1997; Levine, 1989). While carbonylated proteins are believed not to undergo electron reduction, we previously discovered the protein de-carbonylation mechanism, in which carbonyl groups can be eliminated without proteins being degraded (Wong *et al.*, 2008). Major amino acid residues that are susceptible to iron-catalyzed oxidation include proline and arginine, both of which are oxidized to become glutamyl semialdehyde, which contains a carbonyl group (Amici *et al.*, 1989). Glutamyl semialdehyde is further oxidized into glutamic acid (Figure 1).

We previously demonstrated the role of protein carbonylation in ligand/receptor-mediated cell signaling (Wong *et al.*, 2008). We further noted that the kinetics of ligand-mediated protein carbonylation is transient. Typically, in cultured cells, ligands activate the carbonylation of various proteins within 10 min and the activated protein carbonylation reverts to baseline by 30 min. These results suggest that there is a mechanism for the elimination of the formed carbonyls. We named this process

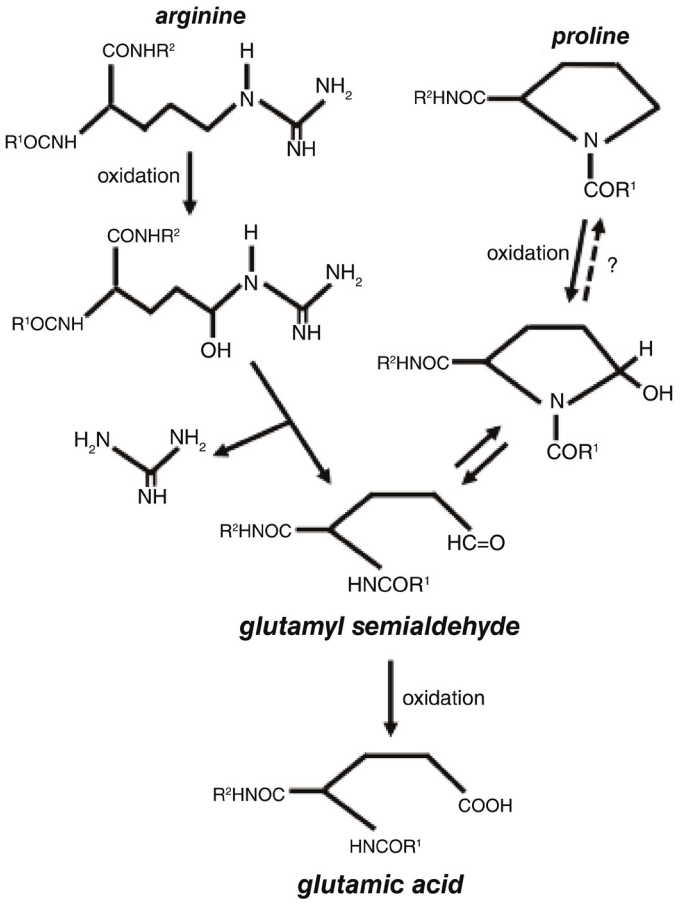

Figure 1. Iron-catalyzed oxidations of arginine and proline residues that result in the formation of glutamyl semialdehyde with a carbonyl group (Amici *et al.*, 1989). Glutamyl semialdehyde is further oxidized into glutamic acid.

"de-carbonylation" (Wong *et al.*, 2008). To understand the mechanism of de-carbonylation, we tested the hypothesis that protein carbonyls may be reduced. We found that the addition of reductants to rat heart homogenates resulted in a decrease in the protein carbonyl content (Wong *et al.*, 2013). By contrast, reductants had no effect on the carbonyl content in purified proteins, suggesting that protein carbonyls are not reduced in the absence of other cellular components. From these results, we hypothesized that cells contain catalysts for the reduction of protein carbonyls. This hypothesis is supported by our results demonstrating that the heating of heart homogenates to inactivate cellular enzymes inhibits the decrease in protein carbonyls *in vitro,* and that knocking down glutaredoxin 1 in the cells inhibits protein de-carbonylation (Wong *et al.*, 2013). We used two-dimensional gel electrophoresis and mass spectrometry to identify proteins that can be de-carbonylated and found that peroxiredoxin 6 (Prx6) is one such protein (Wong *et al.*, 2013).

Since both arginine and proline residues can be oxidized to form glutamyl semialdehyde that can further be oxidized to form glutamic acid, we speculated that arginine, proline, and glutamic acid residues may be interchangeable in the biological system, in a process that resembles site-directed mutagenesis. This article reports that the proline residue 45 of the human Prx6 protein molecule can be converted into glutamic acid in cells, indeed demonstrating the existence of a naturally occurring site-directed mutagenesis/protein engineering-like process that may be regulated by ROS.

Methods

Cell culture and immunoprecipitation

Human pulmonary artery smooth muscle cells (ScienCell Research Laboratories, Carlsbad, CA, USA) grown in 10 cm dishes were serum-starved overnight with 10 ml of 0.01% fetal bovine serum-containing Dulbecco's Modified Eagle's medium (Mediatech, Inc., Manassas, VA, USA) for cell signaling studies. To prepare lysates, the cells were washed with phosphate buffered saline and solubilized with 1 ml of 50 mM Hepes solution (pH 7.4) containing 1% (v/v) Triton X-100, 4 mM EDTA, 1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM tetrasodium pyrophosphate, 2 mM PMSF, 10 µg/mL leupeptin, and 10 µg/mL aprotinin. Cell lysates (1 ml) were immunoprecipitated with the rabbit polyclonal anti-Prx6 antibody (Sigma-Aldrich, St. Louis, MO, USA; Catalogue # P0058; 5 µg) and SureBeads Protein G Magnetic Beads (Bio-Rad Bio-Rad Laboratories, Hercules, CA, USA; 1 mg) for 1 h at room temperature.

Peptide sample preparation

Immunoprecipitation samples were processed with trypsin digestion (12.5 ng/µl) followed by a C18 Zip-tip clean-up (EMD Millipore, Billerica, MA, USA). Tryptic peptide samples were reconstituted in 20 µl of 0.1% formic acid before nanospray liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) analysis was performed.

Nanospray LC/MS/MS analysis

The tryptic peptides mixture from each sample was analyzed using a Thermo Scientific Q-Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Electron, Bremen, Germany) equipped with a Thermo Dionex UltiMate 3000 RSLCnano System (Thermo Dionex, Sunnyvale, CA, USA). Tryptic peptide samples were loaded onto a peptide trap cartridge at a flow rate of 5 µl/min. The trapped peptides were eluted onto a reversed-phase 20-cm C18 PicoFrit column (New Objective, Woburn, MA, USA) using a linear gradient of acetonitrile (3–36%) in 0.1% formic acid. The elution duration was 60 min at a flow rate of 0.3 µl/min. Eluted peptides from the PicoFrit column were ionized and sprayed into the mass spectrometer using a Nanospray Flex Ion Source ES071 (Thermo Scientific, Waltham, MA, USA) under the following settings: spray voltage 1.6 kV and capillary temperature 250°C. The Q Exactive instrument was operated in the data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (m/z 300–2,000) were acquired in the Orbitrap with 70,000 resolution (m/z 200) after the accumulation of ions to a 3×10^6 target value based on predictive AGC from the previous full scan. Dynamic exclusion was set to 20 s. The 15 most intense multiply charged ions ($z \geq 2$) were sequentially isolated and fragmented in the Axial Higher Energy Collision-induced Dissociation (HCD) cell using normalized HCD collision energy at 25% with an AGC target of 1e5 and a maximum injection time of 100 ms at 17,500 resolution. Two independent MS analyses in triplicate (a total of six cell samples) were performed.

LC/MS/MS data analysis

The raw MS files were analyzed using the Thermo Proteome Discoverer 1.4.1 platform (Thermo Scientific, Bremen, Germany) for peptide identification and protein assembly. The raw data files were searched against the human protein sequence database obtained from the NCBI website (https://www.ncbi.nlm.nih.gov) using the Proteome Discoverer software based on the SEQUEST algorithm. The carbamidomethylation of cysteines was set as a fixed modification, and Oxidation and Deamidation Q/N-deamidated (+0.98402 Da), and Pro>Glu (+31.990 Da) were set as dynamic modifications. The minimum peptide length was specified to be five amino acids. The precursor mass tolerance was set to 15 ppm, whereas fragment mass tolerance was set to 0.05 Da. The maximum false peptide discovery rate was specified as 0.01.

Results

Identification of the conversion of proline residues into glutamic acid in Prx6

To identify protein carbonylation sites, we enriched Prx6 by immunoprecipitation from cultured human cells. The Prx6 immunoprecipitation samples were processed for digestion by trypsin and the tryptic peptides were analyzed by nanoLC-MS/MS analysis and protein sequence alignment to identify proline sites conversion into glutamic acid in Prx6. The conversion was identified based on a mass shift of + 31.990 Da at the proline residue (Figures 2A and B). The experiments led to the identification of one specific site at Pro 45 in human Prx6 protein (Figure 2C).

Confirmation of Prx6 peptides containing proline to glutamic acid conversion by MS/MS

We are reasonably confident that the identified mass shift of + 31.990 Da is caused by the conversion of proline into glutamic acid, since the Prx6 was affinity-purified before MS/MS analysis.

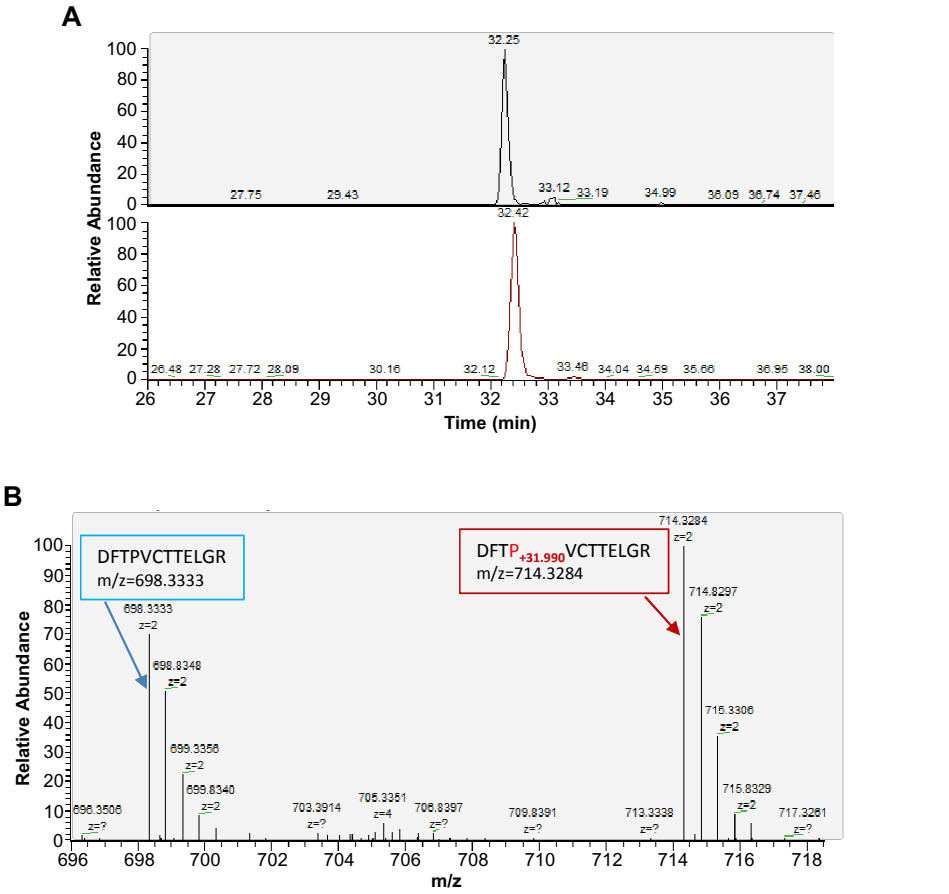

Figure 2. Identification of the conversion of the proline (P) residue at amino acid 45 into glutamic acid (E) in human peroxiredoxin 6 (Prx6). (**A**) Extracted ion chromatograms of Prx6 peptide (DFTP$_{+31.990}$VCTTELGR, +2 charge, m/z=714.33) (top) and its non-conversion counterpart (DFTPVCTTELGR, +2 charge, m/z=698.33) (bottom). Both peptides were eluted at the same retention time and are from affinity-enriched cultured human cell extract using the anti-Prx6 antibody. (**B**) High resolution MS spectra of the co-elution of peptides (DFTP$_{+31.990}$VCTTELGR, +2 charge, m/z=714.33) (right) and its non-conversion counterpart (DFTPVCTTELGR, +2 charge, m/z=698.33) (left). (**C**) Illustration of the identified proline 45 conversion into glutamic acid in cultured human cells (shown in bold red). Sequence areas containing amino acid residues shown in green are detected by LC-MS/MS analysis after trypsin digestion.

Since the conversion of proline into glutamic acid in Prx6 is a novel post-translational modification identified so far, it is desirable to confirm the structure of the identified peptides to ensure that the derived mass shifts of +31.99 Da are caused by the conversion into glutamic acid. MS/MS and HPLC co-elution are gold standards for verifying peptide identification. As demonstrated in Figure 3, both peptides, DFTP+31.990VCTTELGR, +2 charge, m/z=714.33, and its non-conversion counterpart DFTPVCTTELGR, +2 charge, m/z=698.33 were co-eluted with a peak shift of less than 0.2 min. Our result showed that the high resolution MS/MS fragmentation patterns of DFTP+31.990VCTTELGR and its non-conversion counterpart DFTPVCTTELGR peptide were almost identical except the addition of +31.990 Da of fragments that contain the proline 45 residue (Figures 3A and B).

Analysis of the ion intensity of the MS spectra of DFTP+31.990VCTTELGR and its non-conversion counterpart DFTPVCTTELGR peptide (Figure 3C) determined that the proline 45 to glutamic acid conversion occurs in 5–10% of the Prx6 molecule in our samples with a mean of 7.43 ± 1.78% (N=6).

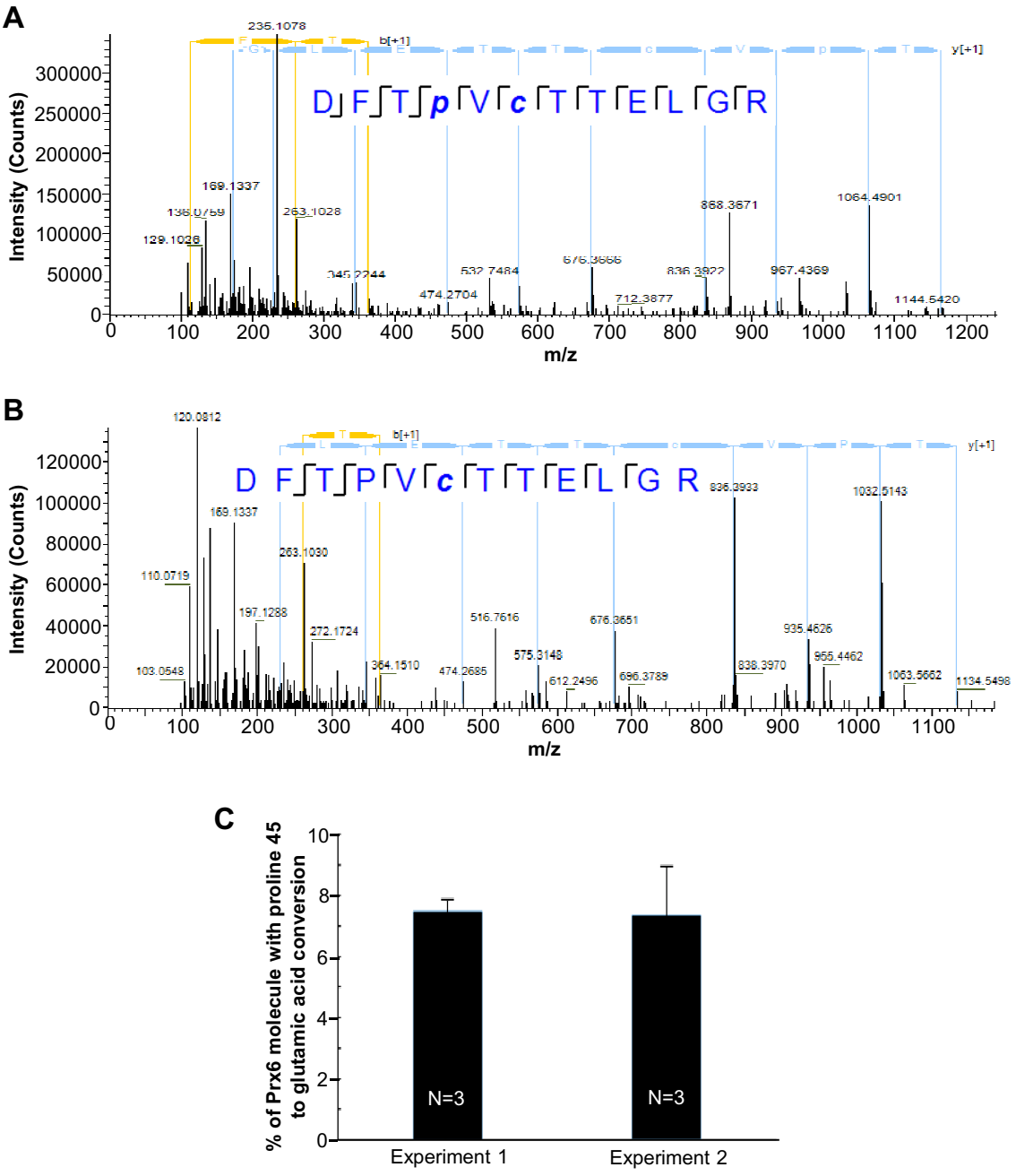

Figure 3. NanoLC-MS/MS verification of the conversion of proline 45 into glutamic acid at Prx6 (DFTP$_{+31.990}$VCTTELGR). (**A**) High resolution MS/MS spectra of peroxiredoxin 6 (Prx6) proline to glutamic acid conversion peptide (DFTP$_{+31.990}$VCTTELGR). (**B**) High resolution MS/MS spectra of Prx6 proline 45 peptide (DFTPVCTTELGR). Spectrum was obtained by LC-MS/MS analysis using the Thermo UltiMate 3000 RSLCnano System and Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer. (**C**) % of Prx6 molecules with the proline 45 conversion into glutamic acid in cultured human cells. Two independent MS analyses in triplicate (a total of six cell samples) were performed.

Discussion

The present study introduces a revolutionizing concept that a protein engineering-like process could occur naturally in the biological system. Specifically, we identified that proline 45 of the Prx6 protein can be converted into glutamic acid. Proline 45 is in the peroxidase catalytic domain (Fisher, 2011; Fisher, 2017), thus this conversion should have functional significance. Future work should identify if this conversion increases, decreases or modifies the catalytic activity of Prx6. Such studies would open up the possibility that proteins with altered amino acid sequences have functional roles in the biological system.

The results from the present study also open up a new mechanism of ROS, indicating that the amino acid conversion, specifically the proline–glutamic acid conversion, is a consequence of oxidative stress mediated by the formation of glutamyl semialdehyde in the process of protein carbonylation. Through glutamyl semialdehyde, other conversions among arginine, proline, and glutamic acid are possible. Since the caged and site-directed production of hydroxyl radicals and carbonyl formation can occur via metal binding to specific sites of the protein structure (Stadtman & Berlett, 1991; Wong *et al.*, 2010), ROS-mediated amino acid conversion may be a tightly regulated process.

Data availability

The raw MS files from the output of the LC/MS/MS are available: doi, 10.17605/OSF.IO/5FN2E and 10.17605/OSF.IO/RP9J8 (Suzuki, 2017a; Suzuki, 2017b).

Author contributions

YJS conceived the study and designed the experiments. JH and YJS carried out the research. JH and YJS prepared the first draft of the manuscript. Both authors were involved in the revision of the draft manuscript and have agreed on the final content.

Competing interests

No competing interests were disclosed.

Grant information

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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PubMed Abstract | Publisher Full Text | Free Full Text

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Current Referee Status: ✔ ✘ ✘

Version 1

Referee Report 28 June 2017

doi:10.5256/f1000research.12281.r23077

✘ **Adelina Rogowska-Wrzesinska** [1], **Michael J. Davies** [2]

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[2] Sydney Medical School, University of Sydney, Sydney, NSW, Australia

General overview:

This manuscript presents an interesting aspect of the effect of ROS on proteins – the possibility of converting one type of amino acid into another one. It briefly describes the idea and presents results of a single mass spectrometry experiment that identifies two forms of a peptide obtained by trypsin digestion of Prx6 protein. One form contains proline residue and the other form contains modified form of the proline residue. The modification mass is +31.990 Da and based on that the authors conclude that oxidative stress can lead to carbonylation of proline and its further conversion to glutamic acid. No evidence is provided to proof the link between protein oxidation, ROS and the conversion of proline to glutamic acid.

Detailed comments:

1. Abstract. Modified proteins are degraded by multiple enzymatic mechanisms not just the proteasome. Well established roles for lysosomes, LON protease and other proteases have been demonstrated.

2. The authors suggest that the Pro and Arg conversion to Glu is “interchangeable”. This statement suggest reversibility of the process, which is clearly not the case – it is a one-way reaction.

3. The conversion of one amino acid into another via oxidative reactions is definitely not a “revolutionizing concept”. It is very well established that His is converted to Asn and Asp, that Trp (and also other amino acids) can be converted to Gly (via side-chain elimination reactions), that Cys can be converted to Ala.

4. Introduction section is written with a focus on authors own work and its relevance to this work is actually not 100% clear. At the same time a lot of information is missing: Have similar processes been observed before (*in vitro* and/or *in vivo*)? Can other amino acids undergo similar conversion processes? What is the state-of-art in this field?

5. Carbonylation is not limited to Pro and Arg ! Unfortunately this is not clear from the abstract or the introduction.

6. Methods are described in a very short form and a number of details are missing e.g. conditions for immunoprecipitation or protein digestion, amount of starting material and material used for LC-MS analysis. RAW files and processed files should be submitted to MS data repository like for example PRIDE archive.

7. It is not clear at all why the cells where starved prior the experiment and how this is linked to oxidative stress and protein carbonylation. No comparison to non-starved cells had been made.

8. It is unclear from the text that the Cys in the DFTPVCTTELGR peptide is modified. Although not stated in the methods section it seems that the samples have been reduced and alkylated because the Proteome Discoverer search parameters included carbamidomethylation of cysteines as a fixed modification.

9. +31.990 is the modification mass of proline residue. Are there any other types of post translational modifications that would result in a similar mass change?

10. Are there any other modifications present in Prx6?

11. The MS/MS spectra in figure 3 is the only evidence of the Pro to Glu conversion. Is this the only spectra that have been observed? According to methods section six samples have been analysed by LC-MS. How many times this peptide was fragmented in each sample?

    Presentation of multiple spectra would increase the credibility of the observation. Additionally the quality of the figure is not very high and it is very difficult to read the masses of the ions present in the spectra. Therefore again submitting the results to a MS data repository would help to validate the quality of the obtained results.

12. The basis of this selective oxidation is not addressed.

13. It is unclear why the authors do not test the functional significance of this modification, if they have already purified the material – it is not a very difficult assay.

14. Additional experiments where cells are collected at different conditions involving oxidative stress would help to provide the link between the carbonylation and conversion of Pro to Glu. For the moment is in not clear if this conversion is driven by oxidative stress or another unknown process.

15. The authors should not quote amino acid conversion levels based on ion intensities to 2 decimal places (7.43 +/- 1.78%). Unlikely to be this accurate.

16. The comments in the Discussion about “altered amino acids having functional roles” betrays a lack of knowledge of the protein oxidation field – this is very well established (e.g. all the work on oxidised Cys residues).

17. Many of the references cited are rather old. The most recent publications is authors own work. The field has moved on since many of these works were published.

18. The authors final statement “ROS-mediated amino acid conversion may be a tightly regulated process” should be tempered (or completely omitted). “ROS” is a very generic term, and the vast majority of oxidants do not show marked residue and site specificity. They are not “tightly

regulated" in the vast majority of cases.

19. No discussion on the limitations of the presented results and conclusions is given.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

We have read this submission. We believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Referee Report 20 June 2017

doi:10.5256/f1000research.12281.r23628

Dolores Pérez-Sala
Biological Research Center (CIB), Spanish National Research Council (CSIC), Madrid, Spain

In their manuscript, Suzuki and Hao report the finding of a peptide in Peroxiredoxin 6 that shows a mass increment of 32 in mass spectrometry analysis. NanoLC-MSMS analysis maps this increment at the site of a proline residue (P45 in the protein). This mass increment is found to affect approximately 7% of the peroxiredoxin 6 protein present in the samples.
In view of these results the authors interpret that proline has suffered an oxidative modification leading to its conversion in glutamic acid.
Our impression is that the information provided is not sufficient to establish this point.
The mass increment of 32 Da could also be due to dihydroxylation of proline, which is a known posttranslational modification.
Please see:
http://web.expasy.org/findmod/findmod_masses.html

Therefore, additional experimental evidence will be required to confirm the authors' conclusion.
Specifically, we would suggest several of the following approaches:
-Synthetize both the peptide with proline and with glutamic acid

-Analyze the two peptides by HPLC. If they separate, do the same type of analysis with the peptides from their samples
-Attempt to oxidize the proline-containing peptide in vitro (or the intact protein) to monitor the changes in proline
-Perform amino acid analysis to confirm the presence of glutamic acid
-Employ other derivatization or detection strategies to confirm the presence of glutamic acid.

Ideally, the modification described could be explored in other cell types under different oxidative conditions.

If the authors cannot obtain an unequivocal confirmation of the presence of glutamic acid, the title of the manuscript and the main interpretations should be changed.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
No

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Referee Report 14 June 2017

doi:10.5256/f1000research.12281.r23475

Joaquim Ros
Department of Basic Medical Sciences, IRB-Lleida (Biomedical Research Institute of Lleida), University of Lleida, Lleida, Spain

The paper submitted by Suzuki and Hao highlights the importance of modifications occurring in proteins as a consequence of oxidative stress. In this particular case, the authors provide data showing that proline residue at position 45 in peroxiredoxin 6 can be converted into glutamic acid residues through glutamyl semialdehyde. The results shown in the paper are well designed, solved and clear. From

technical point of view, the approach is precise and gives the information necessary to draw the conclusions. Nevertheless there are some minor details that this reviewer consider that should be added or corrected in the text:

1. The conversion of proline to glutamic semialdehyde is already known. Since the results show that the carbonyl group is further oxidized to glutamic acid, the authors should add a sentence about how they believe this last step of oxidation occurs.
2. The authors show that P45 is transformed to E (provided a mass increase of 31,990 daltons). Did the authors check (or find) the intermediate form –the glutamic semialdehyde- and if so, to what extent this intermediate is further oxidized to glutamic acid? A brief sentence should be added to the text if they have these data.
3. There is an exciting idea concerning the concept of “naturally occurring protein engineering”. Being this true, do the authors believe that this could be a motor for evolution? Could they add a short comment on that?
4. Finally, I disagree with the use of “interchangeable” in the text. This would induce to think that a protein could have a P or a E or a R in a given position without compromising its function. It is hard to believe that changing an E for an R would result in a neutral consequence. Since the consequences of such change (increase or decrease activity, stability,…) has not been proved in the case of Prx6, it seems reasonable that the term should be removed and simply say that this could be a driving force for evolution, for instance (as suggested above).
5. In the “Discussion” I would suggest the authors to change the sentence starting with “.Such studies would open up…”. I think they can really say “…studies will open up…” and change “…have functional roles…” for “can acquire new functional roles…”. Do the authors agree?

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.