nature portfolio

Peer Review File

Hidden route of protein damage through oxygen-confined photooxidation

Corresponding Author: Professor Duyoung Min

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript described photo-oxidation experiments with maltose-binding protein (MBP) using blue-light (LED) illumination in the presence or absence of O2. The authors report that they have unveiled a novel photo-chemical process that involves site-specific oxidation induced by 'bound' O2 within hydrophobic cavities in the protein. This results in oxidation primarily at these sites. Further experiments are carried out with HeLa cells.
The data - if correct – are novel and intriguing, but these claims for a new mechanism are radical and therefore require a very high level of supporting data, which is the opinion of this reviewer are not currently provided.
The authors should address the following points.
1) The authors claim a new mechanism of 1O2 formation by Trp residues when illuminated with blue light (447 nm). This claim is unusual, because Trp residues (as indicated by the authors) do not absorb in this region of the UV-vis spectrum. Consequently additional data (e.g. 1O2 emission data, D2O experiments) should be included to verify that 1O2 is generated. Extraordinary claims, require extraordinary levels of data to support them.
2) The damage induced on MBP, and also the cellular proteins, is ascribed to 1O2, but this includes modification at residues that do not typically react rapidly with this species (which is usually limited to Trp, Tyr, His, Phe, Met and Cys). Alkyl side chains are not typically oxidized, so the evidence for modification at these residues needs to be verified as coming from 1O2 and not other species. D2O experiments would be helpful.
3) The authors should discuss the potential occurrence of other mechanisms that may help explain the observed selectivity, including electron transfer reactions which can occur over long distance in proteins. Most photochemical processes are not 100% type 1 or type 2, and typically mixtures of both excited state species and radicals are generated. These possibilities are not discounted by the authors.
4) The cell data is highly speculative, which the authors partially admit to, given that many of the most heavily modified proteins are also the most abundant. Consequently the value of these cell experiments to the overall picture is questionable.
5) The mass spectroscopy data appears to be incomplete. There are a number of parameters which would be expected to be provided that are missing including lists of the detected peptides and the sequence coverage, the site occupancy at different sites, and details of the number of independent and technical replicates that have been run. The authors assertions about the selectivity of modification are dependent on searches for +16 species, which are only a limited fraction of the products formed (others include +14, +32, +4 (for Trp) and many others). Failure to quantify these can result in erroneous data with regard to the extent of oxidation as the measurements require details of the total yield of species (parent and oxidized). Similar points can be made about the cell data.
6) Little consideration is given to previous studies that have characterized the products formed by 1O2, some of which can act as signatures of this species. Identification of some of these species might support the authors conclusions as to the role of 1O2.
7) Much of the analysis of the O2 binding pockets depends on analysis of structures that may not reflect those present in solution (e.g. those predicted by AlphaFold or from solid state studies). Experimental data on protein flexibility and motional dynamics could be obtained by use of hydrogen-deuterium exchange mass spectroscopy.
8) The assays used to 'quantify' the various oxidants are all relatively unspecific, and any data obtained using these assays needs to be treated with extreme caution. Even the EPR measurements are not specific, as the oxidation of TEMP to the nitroxide radical is a multiple step process, and (by definition) results in the formation of radical species. TEMP is not a 'spin trapper', as the initial reaction is a molecular process.
9) The authors should provide stronger data to address the argument that the observed selectivity is not merely driven by the position of the Trp residues within a structure by examining other proteins where the Trp residues are in different

environments. Building the case for a novel mechanism on the basis of studies on only a single protein is questionable.

Minor points
The grammar would benefit from editing by a native English speaker.
The number of references (>100 is excessive).

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
The paper by Kim et al. talks about a newly discovered uncoventional mechanism of protein oxidation. Dioxygen molecules routinely find their way into small internal cavities that are often found in protein's hydrophobic core. There they frequently come in contact with Trp residues. If at the same time they are hit by blue light (400-500 nm), O2 becomes converted into highly reactive 1[O2], which promptly oxidizes the surrounding (mostly hydrophobic) sites. The mechanism is relevant for thousands of proteins in those cells that are exposed to sun light (skin, eyes) and is likely implicated in ageing, cancers, etc.

This is a really beautiful paper. It is very well written. Better yet the narration follows the path of the actual discovery. It begins with the first (somewhat serendipitous) results, which initially appear puzzling. Then control experiments, leading to a working hypothesis. Then the hypothesis is tested by a carefully designed set of experiments and calculations, proving itself beyond reasonable doubt. Finally, cell culture experiments complemented with structural and functional bioinformatics analyses demonstrate the general significance of the discovered mechanism. Case closed. The authors employ a powerful battery of different experimental and computational techniques, beginning with single-molecule tweezers and ending with whole-cell proteomic analysis using LC-MS/MS (the entire list is too long to reproduce here).

I am very much in favor of publishing this paper. I would only like to draw the authors' attention to one experimental method, which they might have overlooked. Since O2 molecules are paramagnetic, their presence in the protein's hydrophobic core can be detected by means of paramagnetic enhancement of the protein's site-specific spin relaxation rate (see e.g. Kitahara et al. Detecting O2 binding sites in protein cavities, Scientific Reports 6, 20534 (2016); Teng & Bryant Experimental Measurement of Nonuniform Dioxygen Accessibility to Ribonuclease A Surface and Interior, J. Am. Chem. Soc. 122, 2667 (2000)). In all likelihood such PRE-based mapping would reveal the oxygen's access to the cavities of interest inside the MBP protein. In my opinion it would be sufficient if the authors just mention this possibility as an option for future research in this area.

(Remarks on code availability)
A collection of scripts, well structured and well documented.

Reviewer #3

(Remarks to the Author)
This is an interesting manuscript concerning a new pathway of protein damage by oxygen-confined photooxidation. This pathway consists of three key processes: (1) oxygen binds to the hydrophobic cavities of proteins; (2) light irradiation excites tryptophan (Trp) residues in the cavities and induces tryptophan-mediated generation of singlet oxygen; (3) The single oxygen reacts with nearby amino acids, leading to protein damage. They induced photo-damage of maltose binding proteins (MBP) by blue light and probed protein damage by detecting force-induced unfolding of single MBP using magnetic tweezers. The damaged proteins exhibit distinct mechanical unfolding patterns compared with the WT protein. Additional mechanisms are supported by mass spec analysis, MD simulations, EPR spectroscopy, DFT calculations, and bioinformatics. Overall, this work proposes an innovative concept regarding protein damage, which is supported by carefully designed and executed experiments. Therefore, the manuscript is recommended for publication in NC after careful revision to address the following concerns:

1. Single MBP proteins are conjugated to DNA handles via two SpyCatcher proteins (Fig. 1a). The authors assumed that both SpyCatcher proteins, which are pulled on different sites, remain folded during the pulling experiments with force up to 50 pN. Yet, no supporting experimental data is shown. A control experiment should be performed by pulling only SpyCatcher proteins with the MBP protein replaced by unfolded or synthesized polypeptide to confirm the claimed mechanical stability of SpyCatcher protein. This control is important, given a previous report showing the unfolding of the same protein (Guo, Z. et al, Nanoscale, 2021; DOI: 10.1039/d1nr01907d). The new result may be shown in a supplemental figure.
2. The main text should be carefully revised to enhance the readership, given the multidisciplinary methods and scopes involved in this work. For example, in lines 3-4 on Page 4, the authors first mentioned blue light excitation of Trp residue without proper introduction or citation. Another example. What's the "thermodynamic feasibility" in Line 13, Page 7?
3. How does the protein damage depend on light intensity and frequency?
4. Why does oxygen tend to be trapped in hydrophobic cavities? Due to hydrophobic interactions?
5. Fig. 3f is not understood. The three EPR spectra appear to be similar. Could the difference be highlighted?

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This version of the manuscript is significantly improved over the original version, and the new data included has allowed further clarification of the reactive oxidants involved. The authors have downplayed the role of singlet oxygen, but this is still postulated as a significant species involved in the oxidation of the detected residues. This conclusion is however not supported by the additional experimental data provided in the new version of the manuscript, as the presence of D2O does not give rise to any significant enhancement of damage, as would be expected if this species was involved. A number of previous studies on protein oxidation using other sources of 1O2 have shown marked effects of the presence of D2O on markers of oxidation (see, e.g. PMID: 39299525). Thus the new data provided by the authors does not support the intermediacy of 1O2 to a significant extent, and the corresponding sections of the manuscript should be amended accordingly.

The concept that oxidants (such as 1O2) only modify surface residues is not widely accepted (as suggested by the authors – e.g. at lines 50-51 and 411-412), as the authors of the paper indicated above, and also others, have reported that the extent of oxidation does not correlate with the solvent accessible surface areas of the relevant residues.

Minor point:
The authors state that the HPF assay is highly specific for hydroxyl radicals (line 273). This is incorrect. This probe also reacts with a number of other oxidants including peroxynitrite (see PMID: 34050474) and also peroxidase systems. Some of the commercial vendors of this product (e.g. Thermo Fisher) clearly state this limitation. The authors should note this lack of specificity, as otherwise this (incorrect) concept will be promulgated.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns. I support the publication of this manuscript in Nature Communications!

(Remarks on code availability)

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have made additional changes which address the previously raised points

(Remarks on code availability)

Reviewer #1

This manuscript described photo-oxidation experiments with maltose-binding protein (MBP) using blue-light (LED) illumination in the presence or absence of O2. The authors report that they have unveiled a novel photo-chemical process that involves site-specific oxidation induced by 'bound' O2 within hydrophobic cavities in the protein. This results in oxidation primarily at these sites. Further experiments are carried out with HeLa cells.

The data - if correct – are novel and intriguing, but these claims for a new mechanism are radical and therefore require a very high level of supporting data, which is the opinion of this reviewer are not currently provided.

We sincerely appreciate the Reviewer's time and effort in evaluating our work. The revision suggestions were very helpful in improving our manuscript, and we are grateful for the feedback.

The authors should address the following points.

1) The authors claim a new mechanism of 1O2 formation by Trp residues when illuminated with blue light (447 nm). This claim is unusual, because Trp residues (as indicated by the authors) do not absorb in this region of the UV-vis spectrum. Consequently additional data (e.g. 1O2 emission data, D2O experiments) should be included to verify that 1O2 is generated. Extraordinary claims, require extraordinary levels of data to support them.

Following the Reviewer's suggestion, we also performed the ABDA assay using D_2O, which is commonly used and highly specific for $^1O_2$ detection (*Chem Rev* 124, 9949 (2024), *J Photoch Photobio B* 204, 111787 (2020)). In D_2O, which increases the lifetime of $^1O_2$, Trp exhibited a slightly lower extent of absorbance change compared to H_2O. This result may be attributed to a higher rate of oxidative degradation of Trp by $^1O_2$ due to its increased lifetime (*Biochem Biophys Res Commun* 305, 761-770 (2003)). Nonetheless, the same trend was observed in both D_2O and H_2O—a gradual absorbance change for Trp under blue light, in contrast to no noticeable absorbance changes for other tested amino acids (Fig. 3e).

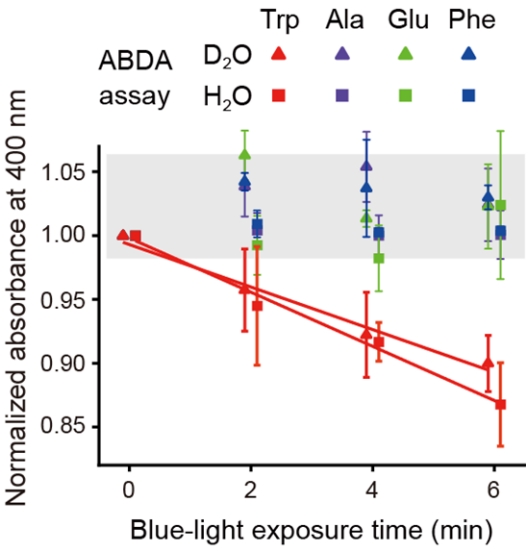


Fig. 3e. ABDA assay for $^1O_2$ detection (n = 3; mean ± SD). The gray-shaded box indicates the region of absorbance for amino acids other than Trp.

These spectrophotometric results (along with the EPR result; see our response to comment 8) indicate that $^1O_2$ can be generated by Trp under blue light. We have included the D_2O result in this revised manuscript and have edited the text accordingly (also attached below). Please refer to our response to comment 3 for a discussion on absorbance in the blue light region.

On page 8, line 34,

> "We confirmed the Trp-mediated $^1O_2$ production using a spectrophotometric assay and EPR spectroscopy (Methods). For Trp dissolved in H_2O containing ABDA as a $^1O_2$ probe[37,38], we observed a gradual decrease in absorbance at 400 nm under blue light irradiation, indicating $^1O_2$-induced ABDA oxidation (Fig. 3e and Supplementary Fig. 19). In contrast, other tested amino acids (aromatic: Phe, charged: Glu, hydrophobic: Ala) did not show noticeable changes in absorbance (Fig. 3e and Supplementary Fig. 19). The same trend was also observed in D_2O, which increases the lifetime of $^1O_2$ (ref. [38]), albeit with a slightly lower extent of change for Trp (Fig. 3e). This result may be attributed to a higher rate of oxidative degradation of Trp by $^1O_2$ due to its increased lifetime[22]. In the EPR measurements using TEMP, commonly used for $^1O_2$ detection[39], we observed the greatest intensity of the characteristic trilinear signals of TEMPO (the oxidized product of TEMP) under the Trp+/light+ condition, compared to the control groups under Trp+/light- or Trp-/light+ conditions (Fig. 3f). The EPR measurement samples contained no other exogenous molecules that could react with TEMP, such as peroxymonosulfate and peracetic acid[39]. Thus, the possibility of false positives is unlikely under our measurement conditions, especially given the suitable controls. The two independent spectrophotometric and EPR experiments indicate that Trp can generate $^1O_2$ under blue light, consistent with the DFT calculations."

2) The damage induced on MBP, and also the cellular proteins, is ascribed to 1O2, but this includes modification at residues that do not typically react rapidly with this species (which is usually limited to Trp, Tyr, His, Phe, Met and Cys). Alkyl side chains are not typically oxidized, so the evidence for modification at these residues needs to be verified as coming from 1O2 and not other species. D2O experiments would be helpful.

Even after re-analyzing the mass spectrometry results (see our response to comment 5), we continue to observe a diversity of oxidized residues, including those with aromatic rings and alkyl side chains (Y, F, H, W, E, A, I, L, T, P, K, S, Q). However, additional spectrophotometric experiments indicate that Trp can also generate type I ROS such as $^{\bullet}OH$, in addition to $^1O_2$ (see our response to comment 3 for more details). Therefore, the diversity of ROS can explain the diversity of oxidized residues—for example, the alkyl side chains, specifically mentioned by the Reviewer, can be relatively easily oxidized by $^{\bullet}OH$. We discussed this point in the revised manuscript.

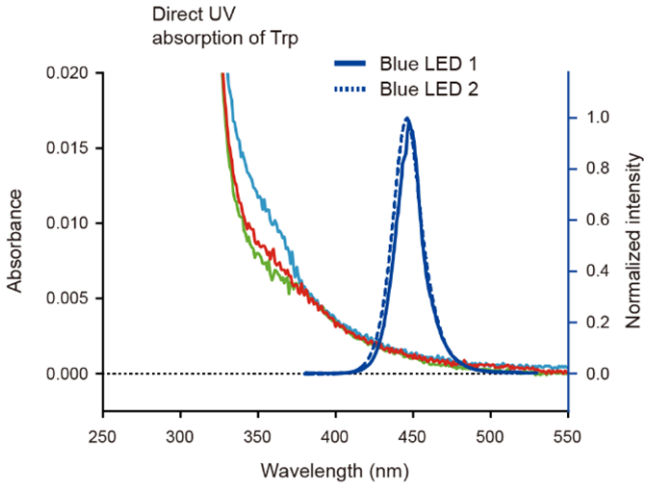
On page 14, line 7,

> "The oxidized residues of MBP identified in the LC-MS/MS analysis encompass a broad range of amino acid types, including those with aromatic rings and alkyl side chains. This result is likely attributed to various ROS, such as $^1O_2$ and $^{\bullet}OH$, possibly generated through multiple photochemical pathways (spin-flip and type I), though it remains unclear which pathway is more dominant. The oxidation sensitivity may also be influenced by the distinct environment within the protein, where the diffusion of ROS is restricted. Specifically, the rugged structural shapes of local protein regions may constrain the diffusion paths and modulate the apparent reaction rates for individual residues[15,48]."

3) The authors should discuss the potential occurrence of other mechanisms that may help explain the observed selectivity, including electron transfer reactions which can occur over long distance in proteins. Most photochemical processes are not 100% type 1 or type 2, and typically mixtures of both excited state species and radicals are generated. These possibilities are not discounted by the authors.

Although $^1O_2$ can be generated by Trp through the spin-flip-based electron transfer pathway (see our response to comment 1), the detection of a relevant absorbance peak in the blue light

region is inherently limited, as this pathway occurs only during transient, short-distance interactions between Trp and O_2. On the other hand, the direct absorption of Trp without O_2 interactions in the UV region shows a tail extending into the blue light region, despite the very low absorption level—only ~1/2000 of the UV peak at 450 nm (Supplementary Fig. 1b). Thus, as the Reviewer noted, we cannot entirely rule out the involvement of the conventional electron transfer (type I) pathway through direct Trp excitation, leading to the generation of type I ROS. However, the conventional energy transfer (type II) pathway would still remain highly unlikely due to the very low, if any, photoluminescence of Trp in the blue light region, as well as the large spectral separation in the resonance energy transfer to O_2, which extends into the IR light region (760–1300 nm) (*Chem Rev* 124, 9949 (2024), *Chem Soc Rev* 49, 5110 (2020), *JACS* 138, 10968 (2016)).



Supplementary Fig. 1b. Absorption spectra of tryptophan (Trp). The absorbance of 30 mM Trp in deionized (DI) water was measured in the range of 250 to 550 nm with 1-nm intervals using a microplate reader. The spectra were corrected by subtracting the blank spectra of DI water. Three different colors indicate triplicate measurements. For comparison, the spectra of light sources used in our study are also shown on the right.

We confirmed Trp-mediated type I ROS generation under blue light, using two widely used spectrophotometric assays: the less specific DHR123 assay, which provides broad detection coverage of type I ROS, including $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ (*JACS* 144, 11326-11337 (2022), *Free Radical Bio Med* 38, 262-270 (2005), *Eur J Biochem* 217, 973-980 (1993)), and the highly specific HPF assay for $^{\bullet}OH$, which is approximately 90–360 times more sensitive than for $O_2^{\bullet-}$ and H_2O_2 (*J of Biol Chem* 278, 3170-3175 (2003)). Please refer the main text and Fig. 3g,h for details (also attached below).

At present, it remains unclear which pathway is more dominant. However, it seems to be clear that Trp can generate various ROS, including both $^1O_2$ and type I ROS, under blue light. We have extended our narrative from Trp-mediated $^1O_2$ generation to Trp-mediated ROS generation—*i.e.*, Trp can generate multiple ROS, not limited to $^1O_2$. We have edited the text accordingly.

[Please refer to the next page for relevant figure panels and text]

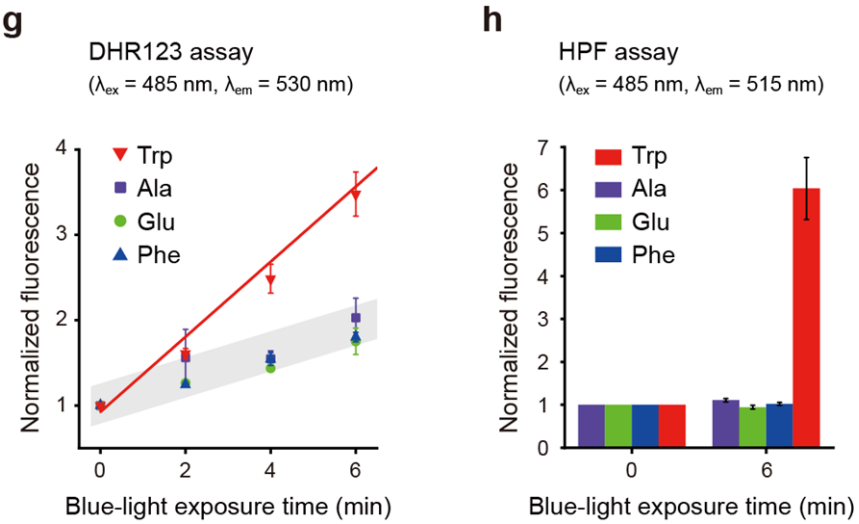


Fig. 3g (left). DHR123 assay for the detection of type I reactive oxygen species (ROS), such as $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ (n = 3; mean ± SD). The gray-shaded box indicates the region of absorbance for amino acids other than Trp. Fig. 3h (right). HPF assay which is more specific to $^{\bullet}OH$ (n = 3; mean ± SD).

On page 9, line 17,

> "The spin-flip-based electron transfer pathway occurs only during transient, short-distance interactions between Trp and O_2, inherently limiting the detection of a relevant absorbance peak in the blue light region. On the other hand, the direct absorption of Trp without O_2 interactions in the UV region shows a tail extending into the blue light region, despite the very low absorption level—only ~1/2000 of the UV peak at 450 nm (Supplementary Fig. 1b). Thus, we cannot entirely rule out the involvement of the conventional electron transfer (type I) pathway through direct Trp excitation. However, the conventional energy transfer (type II) pathway would still remain highly unlikely due to the very low, if any, photoluminescence of Trp in the blue light region, as well as the large spectral separation in the resonance energy transfer to O_2, which extends into the IR light region (760–1300 nm)[37,40,41].
>
> In particular, the type I pathway generates multiple types of ROS, such as $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$. We confirmed Trp-mediated production of type I ROS using two spectrophotometric assays: the less specific DHR123 assay, which provides broad detection coverage of type I ROS, including $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ (refs. [42-44]), and the highly specific HPF assay for $^{\bullet}OH$, which is approximately 90–360 times more sensitive than for $O_2^{\bullet-}$ and H_2O_2 (ref. [45]) (Fig. 3g,h and Methods). In the DHR123 assay, Trp showed a more prominent increase in fluorescence at 530 nm under blue light compared to other tested amino acids, suggesting that type I ROS can also be generated by Trp (Fig. 3g). The spin-flip-based electron transfer pathway also generates $O_2^{\bullet-}$ as an intermediate, which may have contributed to the result of the DHR123 assay. In the HPF assay, only Trp exhibited a dramatic increase in fluorescence at 515 nm under blue light, suggesting that type I ROS, more specifically $^{\bullet}OH$, can also be generated by Trp (Fig. 3h). Therefore, all our experiments and calculations described in this section collectively support the possibility of blue light-induced ROS generation near Trp residues through multiple photochemical processes."

On page 14, line 7,

> "The oxidized residues of MBP identified in the LC-MS/MS analysis encompass a broad range of amino acid types, including those with aromatic rings and alkyl side chains. This result is likely attributed to various ROS, such as $^1O_2$ and $^{\bullet}OH$, possibly

generated through multiple photochemical pathways (spin-flip and type I), though it remains unclear which pathway is more dominant. The oxidation sensitivity may also be influenced by the distinct environment within the protein, where the diffusion of ROS is restricted. Specifically, the rugged structural shapes of local protein regions may constrain the diffusion paths and modulate the apparent reaction rates for individual residues[15,48]."

4) The cell data is highly speculative, which the authors partially admit to, given that many of the most heavily modified proteins are also the most abundant. Consequently the value of these cell experiments to the overall picture is questionable.

There seems to be some misunderstanding regarding the cell-based data. Studying individual proteins can be subjective, as it depends on which and how many proteins are examined. In contrast, the whole-cell proteomic/structural study enables the analysis of overall structure-oxidation trends across a large number of cellular proteins. Although the results were obtained from relatively highly expressed proteins, the 2,915 proteins detected and analyzed are sufficient to assess an overall trend. A key finding from the cell-based study was that the higher the extent of oxidation, the greater the proportion of proteins with Trp-containing cavities large enough to capture O_2. What drives this trend, which is not intuitively obvious? The O_2-confined photooxidation described in this work offers a plausible explanation based on its oxidation mechanism. This observation does not definitively prove our oxidation scenario across a wide range of proteins, which is why we refrained from making a strong claim based solely on the cellular results. However, it aligns with the oxidation model proposed here, based on the results from multiple methods—including single-molecule tweezers, mass spectrometry, MD simulation, DFT calculation, spectrophotometric assays, EPR spectroscopy, and Monte Carlo analysis. As Reviewer #2 noted, we employed this extensive array of methods to integrate all the data and provide robust support for our novel oxidation mechanism, further reinforced by the whole-cell proteomic/structural analysis. We have added a relevant discussion to the Discussion section.

On page 14, line 24,

"Approximately 30–40% of proteins in human and *E. coli* cells possess Trp-containing cavities large enough to capture O_2 molecules. This prevalent structural feature hints at the universality of O_2-confined photooxidation, further supported by the whole-cell proteomic and structural analyses of HeLa cells. Although the results and interpretations are limited to relatively highly expressed proteins (Supplementary Fig. 24b), the number of proteins detected and analyzed (2,915 proteins) is sufficient to assess an overall structure-oxidation trend—the higher the oxidation extent, the greater the proportion of proteins with Trp-containing cavities large enough to capture O_2. What drives this trend, which is not intuitively obvious? The O_2-confined photooxidation described here offers a plausible explanation based on its oxidation mechanism. Hence, the findings—the prevalence of cavity structures in proteins and the observed structure-oxidation correlation—suggest that O_2-confined photooxidation could have a widespread impact on cellular proteins."

5) The mass spectroscopy data appears to be incomplete. There are a number of parameters which would be expected to be provided that are missing including lists of the detected peptides and the sequence coverage, the site occupancy at different sites, and details of the number of independent and technical replicates that have been run. The authors assertions about the selectivity of modification are dependent on searches for +16 species, which are only a limited fraction of the products formed (others include +14, +32, +4 (for Trp) and many others). Failure to quantify these can result in erroneous data with regard to the extent of oxidation as the measurements require details of the total yield of species (parent and oxidized). Similar points can be made about the cell data.

As requested by the Reviewer, all the details—identified peptides, sequence coverage, oxidized sites, modification site occupancy, replicate data, and more—are provided in Source Data for MBP and CA II. CA II is an additional suitable protein we selected and studied in this revision (see our response to comment 9 for more details).

We re-analyzed the mass spectrometry (MS) data of MBP (as well as CA II), with an expanded search list of oxidation species, including +16, +32, +48, +14, and +4 (Supplementary Table 1, also attached below; *Nat Commun* 15, 4025 (2024), *Environ Sci Technol* 54, 14403-14412 (2020)). These selected mass shifts include the major oxidation species, specifically highlighted by the Reviewer. However, we did not include all possible mass shifts for the following reasons: (1) Increasing the number of modifications considered in the search could decrease the number of identifications, potentially resulting in the loss of true oxidized sites. This trade-off has been discussed in previous studies (*Proteomics* 10, 671-686 (2010), *Mass Spectrom Rev* 34, 133-147 (2015)). The vast increase in the search space can sometimes limit the power of MS analysis, particularly when mapping oxidized locations within a single protein. (2) In our study, the purpose of the MS analysis is not to identify all possible oxidative modifications but to examine overall trends in oxidized locations within a single protein—specifically, (i) how frequently the oxidized residues are found within the protein core region, and (ii) how closely they are located to Trp residues.

The same positional trends, which were observed only for +16 in the original manuscript, were also observed for the expanded search list (Figs. 2 and 4, with some of results highlighted below). First, a large portion of the oxidized residues in MBP is located in the protein interior, accounting for 64% of their side chain atoms. Second, Trp is the closest residue type to the oxidized residues, with an exceptionally low occurrence probability of 0.2% for such proximity by random chance. CA II also exhibits similar trends (see our response to comment 9 for more details). These results support the overall narrative of our work.

For the whole-cell MS data, a broader range of mass shifts was already included in the original manuscript's analyses (Supplementary Table 1; *Nat Commun* 15, 4025 (2024)) for the following reasons: (1) The cellular environment is much more complex than the *in vitro* environment of a single protein, potentially leading to a greater diversity of oxidative modifications. (2) The purpose of the whole-cell MS analysis is not to identify specific oxidized locations but to examine overall structure-oxidation trends across a large number of cellular proteins—specifically, whether and how Trp-containing cavities large enough to capture O_2 correlate with the overall extent of protein oxidation (see our response to comment 4 for details).

Name	**Sites**	**Mass**
Single protein (MBP and CA II)[3,4,a]		
Oxidation	MFHILVWYADEKNPQRST	15.994915
Dioxidation	MFHWY	31.989829
Trioxidation	FHWY	47.984744
Carbonylation	HILVYEKPQR	13.979265
Trp to kynurenine	W	3.994915
Whole cell (HeLa cell)[3,b]		
Oxidation	MFHILVWYADEKNPQR	15.994915
Dioxidation	MFWYC	31.989829
Trioxidation	FWYC	47.984744
Carbonylation	ILVEKPQR	13.979265
Arg deguanidation	R	-43.053433
His to Asp	H	-22.031969
His to Asn	H	-23.015984
His – 10	H	-10.0320
His + 5	H	4.9735
Decarboxylation	DE	-30.010565

CO loss	DE	-27.994915
CO_2 loss	DE	-43.989829

Supplementary Table 1. Search lists of oxidative modifications in LC-MS/MS. Cysteine was excluded from the search for single proteins because it is not present in the sequence of MBP and was not detected in CA II.

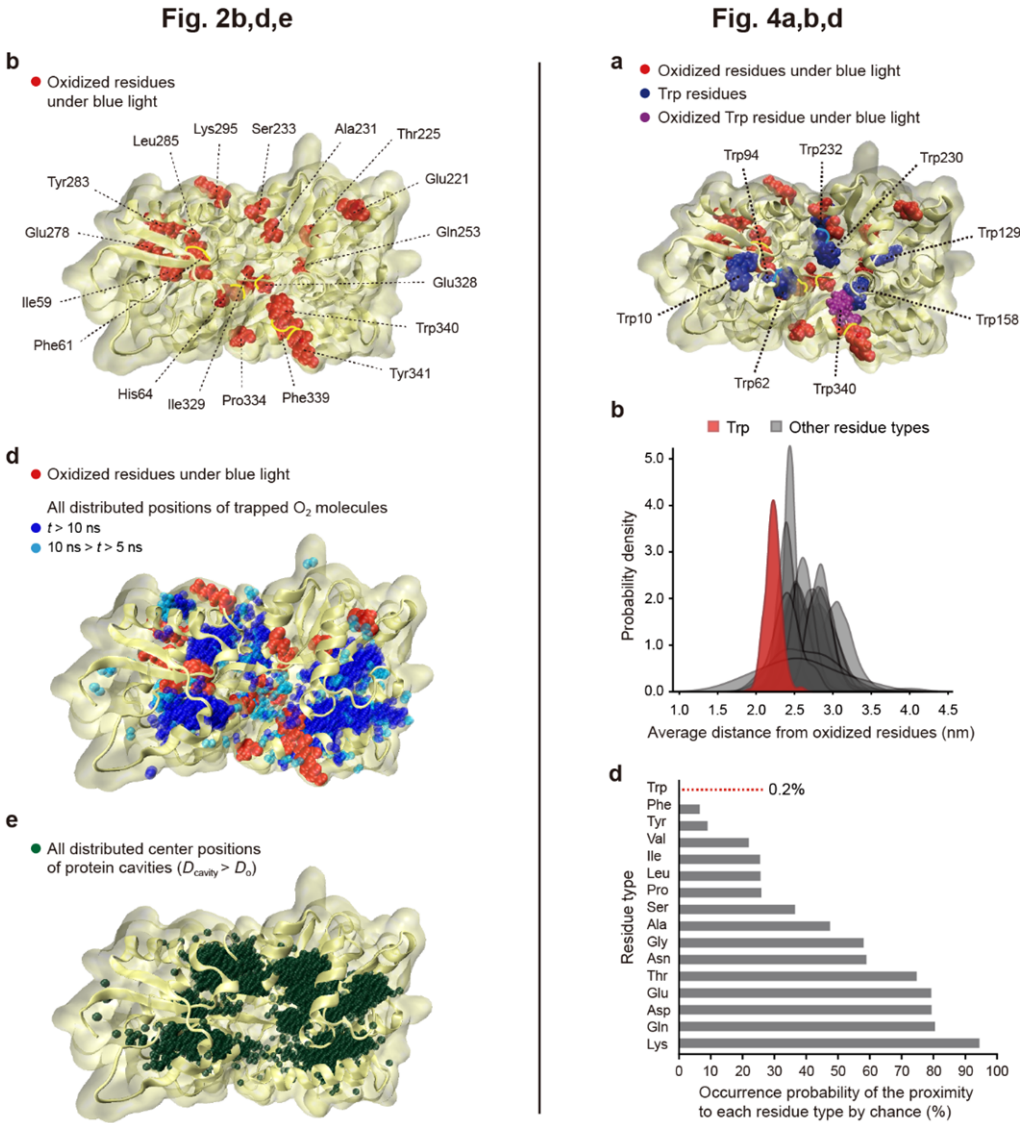


Fig. 2b,d,e (left). (b) Structural distribution of the oxidized residues. (d) All distributed positions of trapped O_2 molecules in simulation #1. Only the O_2 molecules trapped for a specific time range (t) are represented. (e) All distributed center positions of protein cavities with $D_{cavity} > D_o$ in simulation #1. The D_{cavity} and D_o represent the diameter of cavities and the kinetic diameter of O_2 (3.46 Å), respectively. Fig. 4a,b,d (right). (a) Distribution of all tryptophan (Trp) residues and oxidized residues in maltose-binding protein (MBP). The residue numbers indicate those following the signal peptide sequence. (b–d) Monte Carlo analysis for the proximity between Trp and oxidized residues (see Methods). The panels b shows the probability density distributions of average distance from oxidized residues to each residue type. The data for other residue types in panel b are distinguishably presented in Supplementary Fig. 20. The panel d shows the occurrence probabilities of the proximity by chance for all other residue types, which have a larger number of residues than the total number of the benchmark Trp residues in MBP.

6) Little consideration is given to previous studies that have characterized the products formed by 1O2, some of which can act as signatures of this species. Identification of some of these species might support the authors conclusions as to the role of 1O2.

It is uncertain to us which studies/products the Reviewer is referring to. However, two independent measurements using a spectrophotometric assay (ABDA assay) and EPR spectroscopy (using TEMP)—both widely used for $^1O_2$ detection (*Chem Rev* 124, 9949 (2024), *J Photoch Photobio B* 204, 111787 (2020), *PNAS* 120, e2305706120 (2023))—provided results consistent with $^1O_2$ generation. Please refer to our responses to comments 1 and 8 for more details.

7) Much of the analysis of the O2 binding pockets depends on analysis of structures that may not reflect those present in solution (e.g. those predicted by AlphaFold or from solid state studies). Experimental data on protein flexibility and motional dynamics could be obtained by use of hydrogen-deuterium exchange mass spectroscopy.

All relevant analyses and data for MBP and CA II—O_2-binding areas, protein cavity areas, and Monte Carlo results—were derived from MD simulation trajectories, which inherently reflect the protein's flexibility and motional dynamics. Despite being a computational approach, the locations of the O_2-binding areas closely match the protein cavities large enough to capture O_2. This positional match supports the validity of the simulations and relevant analyses, as it is unlikely to be merely a coincidence. As suggested by Reviewer #2, we have added a discussion about future research directions, specifically the potential use of PRE-based spectroscopy for identifying O_2-binding sites.

On page 13, line 34,

> "The O_2-trapping areas of the model proteins MBP and CA II were identified based on MD simulation trajectories, reflecting the protein's flexibility and motional dynamics. The locations of these O_2-trapping areas closely match the protein cavities large enough to capture O_2. While the positional match supports the validity of the simulations and relevant analyses, a well-designed spectroscopic approach could experimentally locate prominent O_2-trapping sites. Since O_2 molecules are paramagnetic, their presence in the protein core region can be detected via paramagnetic enhancement of the protein's site-specific spin relaxation rate[23-26].

Second, the protein's flexibility and motional dynamics were not considered in the whole-cell proteomic/structural analyses, as these analyses were based on 'static' protein structures that were determined experimentally or predicted by AlphaFold. However, this approach is not problematic and is practically viable for the following reasons: (1) For the cellular proteins data, we did not analyze O_2-binding areas, as that was not the focus of the cellular data analyses. (2) Our goal in analyzing the cellular data was to investigate overall structure-oxidation trends across a large number of cellular proteins (see our response to comment 4 for more details). This can only be achieved using 'static' protein structures, as it is currently impossible to consider the flexibility of all 2,915 proteins detected in the cellular experiments within a reasonable time frame.

8) The assays used to 'quantify' the various oxidants are all relatively unspecific, and any data obtained using these assays needs to be treated with extreme caution. Even the EPR measurements are not specific, as the oxidation of TEMP to the nitroxide radical is a multiple step process, and (by definition) results in the formation of radical species. TEMP is not a 'spin trapper', as the initial reaction is a molecular process.

As the Reviewer pointed out, the original version of Fig. 1f, in which the data are labeled as 'ROS type (assay type)', could be misleading, as if each assay is 100% specific to a certain ROS. We have removed the ROS types, so that only the assay types are now designated for each data set without specifying the ROS types.

Additionally, for the newly obtained data using the DHR123 and HPF assays, we cautiously described the results in terms of specificity. On page 9, line 28,

> "…We confirmed Trp-mediated production of type I ROS using two spectrophotometric assays: the less specific DHR123 assay, which provides broad detection coverage of type I ROS, including $O_2^{\bullet-}$, H_2O_2, and $^{\bullet}OH$ (refs. [42-44]), and the highly specific HPF assay for $^{\bullet}OH$, which is approximately 90–360 times more sensitive than for $O_2^{\bullet-}$ and H_2O_2 (ref. [45]) (Fig. 3g,h and Methods)…"

We have responded to the Reviewer's comment on the EPR measurements as follows:

(1) On page 9, line 9, we removed the term 'spin trapper' from the main text, revising the phrase '…a commonly used $^1O_2$ spin trapper…' to '…commonly used for $^1O_2$ detection[37]…'.

(2) TEMP can be less specific to $^1O_2$ in more complex catalytic oxidation systems. However, our system is much simpler, containing only TEMP and Trp, with no other exogenous molecules that could react with TEMP, such as peroxymonosulfate (PMS) and peracetic acid (PAA) (*PNAS* 120, e2305706120 (2023)). To check reproducibility, we purchased fresh TEMP and measured the EPR spectra in triplicate. We observed results similar to those in the original manuscript (Fig. 3f; also attached below). Compared to the control groups (Trp+/light- or Trp-/light+), the experimental group under the Trp+/light+ condition showed the greatest intensity of the characteristic trilinear signals of TEMPO, the oxidized product of TEMP. This EPR result, together with the ABDA assay in H_2O or D_2O (see our response to comment 1)—both widely used for $^1O_2$ detection (*Chem Rev* 124, 9949 (2024), *J Photoch Photobio B* 204, 111787 (2020), *PNAS* 120, e2305706120 (2023))—supports that Trp can generate $^1O_2$ under blue light irradiation.

On page 9, line 8,

> "…In the EPR measurements using TEMP, commonly used for $^1O_2$ detection[39], we observed the greatest intensity of the characteristic trilinear signals of TEMPO (the oxidized product of TEMP) under the Trp+/light+ condition, compared to the control groups under Trp+/light- or Trp-/light+ conditions (Fig. 3f). The EPR measurement samples contained no other exogenous molecules that could react with TEMP, such as peroxymonosulfate and peracetic acid[39]. Thus, the possibility of false positives is unlikely under our measurement conditions, especially given the suitable controls. The two independent spectrophotometric and EPR experiments indicate that Trp can generate $^1O_2$ under blue light, consistent with the DFT calculations."

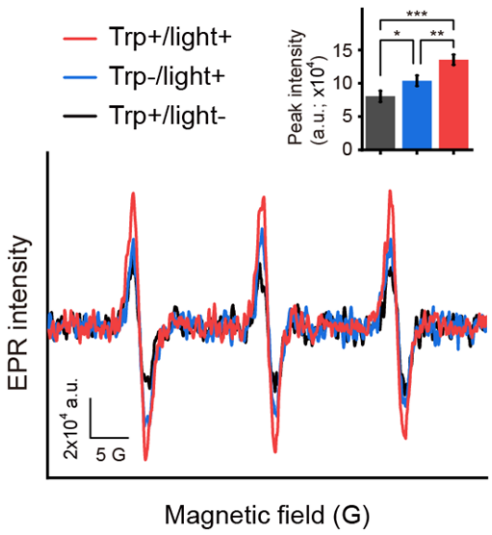


*Fig. 3f. Electron paramagnetic resonance (EPR) measurement using TEMP for $^1O_2$ detection. The EPR spectra are averaged from triplicates for each condition and are expressed in arbitrary unit (a.u.). The inset shows the EPR intensity for each condition (n = 3; mean ± SD). One-way ANOVA with post-hoc Tukey HSD test (*** for p < 0.001, ** for p < 0.01, and * for p < 0.05).*

9) The authors should provide stronger data to address the argument that the observed selectivity is not merely driven by the position of the Trp residues within a structure by examining other proteins where the Trp residues are in different environments. Building the case for a novel mechanism on the basis of studies on only a single protein is questionable.

In response to the Reviewer's suggestion, we further investigated the observed residue selectivity using an additional suitable protein. We selected carbonic anhydrase II (CA II) because it has cavity structures that can accommodate O_2 molecules, as well as several Trp residues in its interior. Additionally, the protein purification process, often a challenge, was already well-established for CA II since our co-author has been studying its structural dynamics and function (*Nat Commun* 11, 4557 (2020), *PNAS* 113, 5257-5262 (2016)). Thus, CA II was also easily accessible experimentally. In conclusion, the results for CA II are consistent with those for MBP. Please refer to the text and figure below for details, which has been added to the revised manuscript.

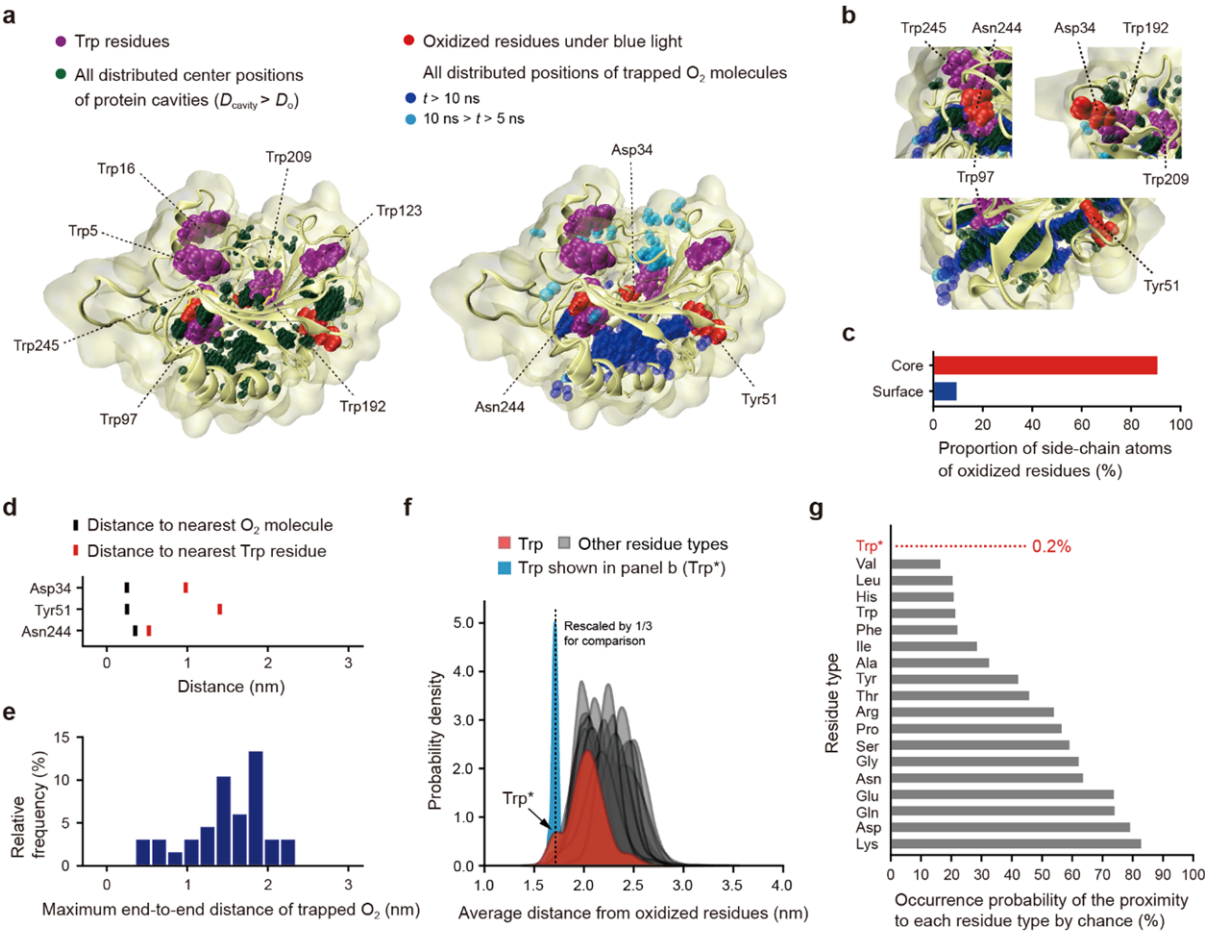


Fig. 5. Positional correlation between Trp and oxidized residues in CA II. (a) Structural distribution of oxidized residues, tryptophan (Trp) residues, protein cavities, and trapped O_2 molecules in carbonic anhydrase II (CA II). Only the O_2 molecules trapped for a specific time range (t) are represented. The D_{cavity} and D_o represent the diameter of cavities and the kinetic diameter of O_2 (3.46 Å), respectively. (b) Close-up views of the regions surrounding the oxidized residues. (c) Proportion of oxidized residues' side-chain atoms located in the protein core and surface regions. (d) Minimum distances from oxidized residues to trapped O_2 molecules or Trp residues. (e) Maximum end-to-end distances of trapped O_2

molecules within the protein obtained from molecular dynamics (MD) simulations. (f,g) Monte Carlo analysis for the proximity between Trp and oxidized residues (see Methods). The panel f shows the probability density functions of average distance from oxidized residues to each residue type (PDF_{avg}). The data for other residue types in panel f are distinguishably presented in Supplementary Fig. 21b. Trp represents a subset of Trp residues (Trp97, Trp192, Trp209, and Trp245), which are responsible for the leftmost peak in the PDF_{avg} for Trp (see the main text for more details). The PDF_{avg} shown in blue is constructed from the sampling of Trp*. The panel g shows the occurrence probabilities of the proximity by chance for all residue types, which have a larger number of residues than the number of benchmark Trp* residues.*

On page 11, line 4,

> "The positional correlation between Trp and oxidized residues was also examined using an additional suitable protein. We selected carbonic anhydrase II (CA II) because it has cavity structures that can accommodate O_2 molecules, albeit to a lesser extent than MBP, as well as several Trp residues in its interior (Fig. 5a and Methods). Indeed, the positions of trapped O_2 molecules, which closely interact with CA II within 3.5 Å over 10 ns (or 5 ns), largely overlap with the protein cavity areas, both analyzed from a 1-μs MD trajectory (Fig. 5a,b and Methods). Additionally, all three oxidized residues under blue light, identified by LC-MS/MS analysis, are predominantly located in the protein core region, with 91% of their side chain atoms residing in this area (Fig. 5a–c, Supplementary Fig. 21a, Supplementary Table 1, and Methods). The oxidized residues are in close proximity to both the trapped O_2 molecules (<3.5 Å and >10 ns) and Trp residues, with minimum distances of 0.25–0.35 nm and 0.5–1.4 nm, respectively (Fig. 5d). Moreover, the range of proximity to Trp falls within the maximum end-to-end distances of trapped O_2 (and roughly ROS as well; see above), reaching up to ~2.3 nm (Fig. 5e).
>
> Similar to our analysis of MBP, we performed Monte Carlo analysis on a 1-μs MD trajectory of CA II to evaluate how unlikely the proximity of oxidized residues to Trp is by random chance (Methods). The identification of only three oxidized residues may indicate that only a small number of Trp residues are involved in residue oxidation in CA II. This conjecture is reasonable because unlike in MBP, certain Trp residues (Trp5, Trp16, and Trp123) in CA II are relatively distant from the trapped O_2 molecules and the cavity areas (Fig. 5a). Indeed, when sampling from all Trp reisudes (n = 7), the overall proximity between Trp and oxidizied residues is not particularly prominent, comparable to other types of hydrophobic residues (Fig. 5f and Supplementary Fig. 21b). However, there is an additional, remarkably close peak in the PDF_{avg} for Trp, which exactly matches the peak of the PDF_{avg} for Trp*, the other group of Trp residues (Trp97, Trp192, Trp209, and Trp245) (Fig. 5f). These Trp residues are the closest to the oxidized residues and are also close to the trapped O_2 molecules and the cavity areas (Fig. 5a,b). Notably, the proximity of oxidized residues to Trp* has an exceptionally low probability of occurring by chance (0.2%), consistent with observations in MBP (Fig. 5f,g, Supplementary Fig. 21c, and Methods). These results with CA II further support the role of Trp as a key residue in ROS generation and nearby residue oxidation."

Minor points

The grammar would benefit from editing by a native English speaker.

We have reviewed the entire text and made necessary edits, correcting all typos and grammatical errors we identified. Please refer to the revised text with track changes.

The number of references (>100 is excessive).

We have reduced the total number of references from 118 to 102 without compromising the integrity of the manuscript. The reference list now includes 58 references for the main text and 44 for the Methods section, both of which we consider essential. The relatively

large number of references in the Methods section reflects the wide range of methods used in this manuscript. Since *Nature Communications* has published papers with over 100 references in some cases (as we found online), we hope our relatively long reference list will also be acceptable."

Reviewer #2

The paper by Kim et al. talks about a newly discovered uncoventional mechanism of protein oxidation. Dioxygen molecules routinely find their way into small internal cavities that are often found in protein's hydrophobic core. There they frequently come in contact with Trp residues. If at the same time they are hit by blue light (400-500 nm), O2 becomes converted into highly reactive 1[O2], which promptly oxidizes the surrounding (mostly hydrophobic) sites. The mechanism is relevant for thousands of proteins in those cells that are exposed to sun light (skin, eyes) and is likely implicated in ageing, cancers, etc.

This is a really beautiful paper. It is very well written. Better yet the narration follows the path of the actual discovery. It begins with the first (somewhat serendipitous) results, which initially appear puzzling. Then control experiments, leading to a working hypothesis. Then the hypothesis is tested by a carefully designed set of experiments and calculations, proving itself beyond reasonable doubt. Finally, cell culture experiments complemented with structural and functional bioinformatics analyses demonstrate the general significance of the discovered mechanism. Case closed. The authors employ a powerful battery of different experimental and computational techniques, beginning with single-molecule tweezers and ending with whole-cell proteomic analysis using LC-MS/MS (the entire list is too long to reproduce here).

I am very much in favor of publishing this paper. I would only like to draw the authors' attention to one experimental method, which they might have overlooked. Since O2 molecules are paramagnetic, their presence in the protein's hydrophobic core can be detected by means of paramagnetic enhancement of the protein's site-specific spin relaxation rate (see e.g. Kitahara et al. Detecting O2 binding sites in protein cavities, Scientific Reports 6, 20534 (2016); Teng & Bryant Experimental Measurement of Nonuniform Dioxygen Accessibility to Ribonuclease A Surface and Interior, J. Am. Chem. Soc. 122, 2667 (2000)). In all likelihood such PRE-based mapping would reveal the oxygen's access to the cavities of interest inside the MBP protein. In my opinion it would be sufficient if the authors just mention this possibility as an option for future research in this area.

Reviewer #2 (Remarks on code availability):
A collection of scripts, well structured and well documented.

We sincerely appreciate the Reviewer for their time and effort in evaluating our work (and for the positive evaluation as well!). In response to this comment, we have added a discussion on the mentioned point, citing relevant references:

On page 13, line 34,

> "The O_2-trapping areas of the model proteins MBP and CA II were identified based on MD simulation trajectories, reflecting the protein's flexibility and motional dynamics. The locations of these O_2-trapping areas closely match the protein cavities large enough to capture O_2. While the positional match supports the validity of the simulations and relevant analyses, a well-designed spectroscopic approach could experimentally locate prominent O_2-trapping sites. Since O_2 molecules are paramagnetic, their presence in the protein core region can be detected via paramagnetic enhancement of the protein's site-specific spin relaxation rate[23-26].

This comment was helpful in improving our manuscript. Thank you very much!

Reviewer #3

This is an interesting manuscript concerning a new pathway of protein damage by oxygen-confined photooxidation. This pathway consists of three key processes: (1) oxygen binds to the hydrophobic cavities of proteins; (2) light irradiation excites tryptophan (Trp) residues in the cavities and induces tryptophan-mediated generation of singlet oxygen; (3) The single oxygen reacts with nearby amino acids, leading to protein damage. They induced photo-damage of maltose binding proteins (MBP) by blue light and probed protein damage by detecting force-induced unfolding of single MBP using magnetic tweezers. The damaged proteins exhibit distinct mechanical unfolding patterns compared with the WT protein. Additional mechanisms are supported by mass spec analysis, MD simulations, EPR spectroscopy, DFT calculations, and bioinformatics. Overall, this work proposes an innovative concept regarding protein damage, which is supported by carefully designed and executed experiments. Therefore, the manuscript is recommended for publication in NC after careful revision to address the following concerns:

We sincerely appreciate the Reviewer for their time and effort in evaluating our work, as well as for their positive feedback. We also found the revision suggestions helpful in improving our manuscript. Thank you very much!

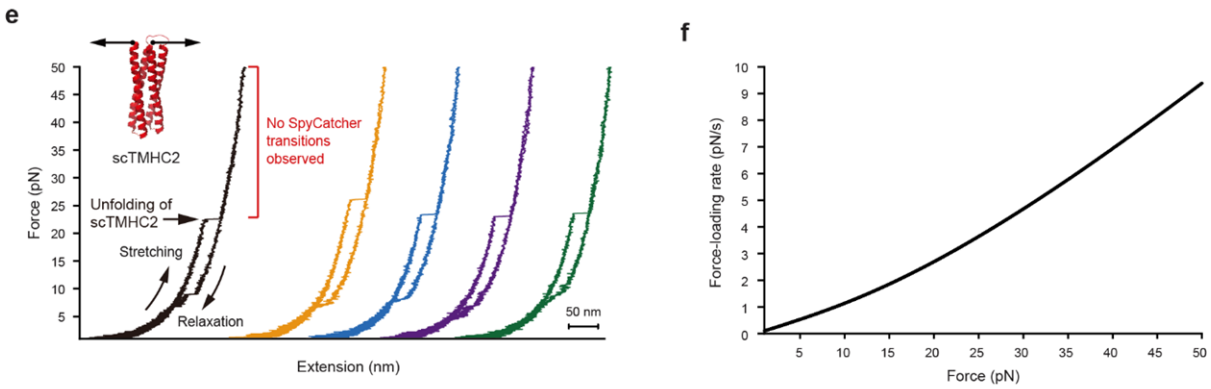
1. Single MBP proteins are conjugated to DNA handles via two SpyCatcher proteins (Fig. 1a). The authors assumed that both SpyCatcher proteins, which are pulled on different sites, remain folded during the pulling experiments with force up to 50 pN. Yet, no supporting experimental data is shown. A control experiment should be performed by pulling only SpyCatcher proteins with the MBP protein replaced by unfolded or synthesized polypeptide to confirm the claimed mechanical stability of SpyCatcher protein. This control is important, given a previous report showing the unfolding of the same protein (Guo, Z. et al, Nanoscale, 2021; DOI: 10.1039/d1nr01907d). The new result may be shown in a supplemental figure.

We understand the Reviewer's concern about the possibility of structural transitions in SpyCatcher that might have affected our single-molecule experiment results. However, in our single-molecule setup, we do not observe these transitions due to the high force-loading rate used, as explained in the following paragraphs.

As this Reviewer may already know, the force-loading rate is a crucial factor in determining the force range for molecular transitions. Specifically, the higher the loading rate, the higher the unfolding forces. In the mentioned previous report (Guo, Z. et al, Nanoscale, 2021; DOI: 10.1039/d1nr01907d), the loading rate was set at a constant 0.5 pN/s, at which SpyCatcher exhibits structural transitions within a force range of 30–40 pN. In contrast, in our current single-molecule setup, the magnet speed is set at a constant 0.3 mm/s, rather than the loading rate (just limited by instrumentation). In this case, the force-loading rate increases as a function of force (see the plot of force-loading rate *vs.* force below). As a result, the loading rate in our setup reaches 5–7 pN/s at around 30–40 pN, where the structural transitions of SpyCatcher were observed in the previous work (Guo, Z. et al, Nanoscale, 2021; DOI: 10.1039/d1nr01907d). This loading rate of 5–7 pN/s is approximately 10–14 times higher than the 0.5 pN/s used in the previous work. Consequently, due to the much higher loading rate in our experiments, SpyCatcher transitions should occur at forces higher than 30–40 pN, likely above 50 pN.

To support this argument, we used a different but more suitable protein as a single-molecule 'fingerprint' protein, similar to the approach in the previous work (Guo, Z. et al, Nanoscale, 2021; DOI: 10.1039/d1nr01907d). Since the unfolding forces of our model protein, MBP, are broadly distributed from 5 to 50 pN (Supplementary Fig. 3c), we employed a structurally simpler protein, scTMHC2, which unfolds in a narrower force range of 14–22 pN (eLife 12:e85882, 2023), clearly separated from the force range of SpyCatcher transitions (30–40 pN or above). This unfolding pattern of scTMHC2 allows us to detect any potential SpyCatcher transitions up to 50 pN. In this experimental design, scTMHC2 serves as a single-molecule 'fingerprint' protein, while the SpyCatcher transitions are the target signals we aim to observe. As expected from the reasoning above, we did not detect any additional transitions

up to 50 pN (see below), suggesting that the SpyCatcher transitions would occur above 50 pN. We have included this result, along with the loading-rate plot for our single-molecule setup, in Supplementary Fig. 3.



Supplementary Fig. 3e,f. (e) Representative force-extension curves showing the unfolding of scTMHC2. The range of its unfolding forces is 14–22 pN (ref. 1), clearly separated from the force range of SpyCatcher's structural transitions, 30–40 pN (ref. 2). In our tweezer setup, no additional transitions up to 50 pN were detected, suggesting that SpyCatcher transitions would occur above 50 pN. This result is likely due to the high force-loading rates shown in panel f, as high loading rates increase the unfolding forces. (f) Force-loading rate as a function of force. In our tweezer setup, the magnet speed is set at a constant 0.3 mm/s, rather than the loading rate. In this case, the force-loading rate increases as a function of force. As a result, the loading rate in our setup reaches 5–7 pN/s at around 30–40 pN, where the structural transitions of SpyCatcher were observed in a previous work[2]. This loading rate of 5–7 pN/s is approximately 10–14 times higher than the 0.5 pN/s used in the previous work. Consequently, due to the much higher loading rate in our experiments, SpyCatcher transitions should occur at forces higher than 30–40 pN, likely above 50 pN.

2. The main text should be carefully revised to enhance the readership, given the multidisciplinary methods and scopes involved in this work. For example, in lines 3-4 on Page 4, the authors first mentioned blue light excitation of Trp residue without proper introduction or citation. Another example. What's the "thermodynamic feasibility" in Line 13, Page 7?
We have reviewed the entire text and made the necessary edits to enhance readability for a broader audience. Please refer to the revised text with track changes. Below are our responses to the specific points raised by the Reviewer.

On page 4, line 4, we revised from

> "...The conversion to $^1O_2$, a potent ROS, is mediated by the blue-light excitation of a molecular system, comprising the captured O_2 and a nearby tryptophan (Trp) residue."

to

> "…The generation of ROS is primarily mediated by the blue light excitation of photosensitizing tryptophan (Trp) residues near the captured O_2 molecules."

Here, the term 'ROS' was already defined in a previous paragraph. This relevant part of the Introduction serves as a brief summary of our work, highlighting key results to engage readers and encourage further reading. Citations are not necessary here, as the text directs readers to the main content for details. We made a slight edit to the sentence, as shown above, and also added the term 'photosensitizing' to better convey the potential for light-mediated conversion from O_2 to ROS.

On page 7, line 14, we revised from

> "…The sustained and close bindings between MBP and O_2 molecules were observed in three 1-μs MD trajectories (Fig. 2c and Methods). The O_2 positions during the

binding times are predominantly distributed inside the protein with thermodynamic feasibility (Fig. 2d, Extended Data Fig. 3, Supplementary Video 1, and Methods)…"

to

"…Sustained and close interactions between MBP and O_2 within 3.5 Å for over 10 ns (or 5 ns) were observed in three 1-μs MD trajectories (Fig. 2c), where O_2 molecules were predominantly located inside the protein (Fig. 2d, Supplementary Figs. 7 and 8, and Supplementary Video 1). The O_2-trapping events were also found to be energetically feasible, based on free energy calculations (Supplementary Figs. 9–11 and Methods)…"

3. How does the protein damage depend on light intensity and frequency?

According to the proposed mechanism of O_2-confined photooxidation, the protein damage is expected to depend on the frequency and intensity of light—*i.e.*, protein damage will be more significantly induced under blue light irradiation. This expectation is confirmed by the data shown in Fig. 1e.

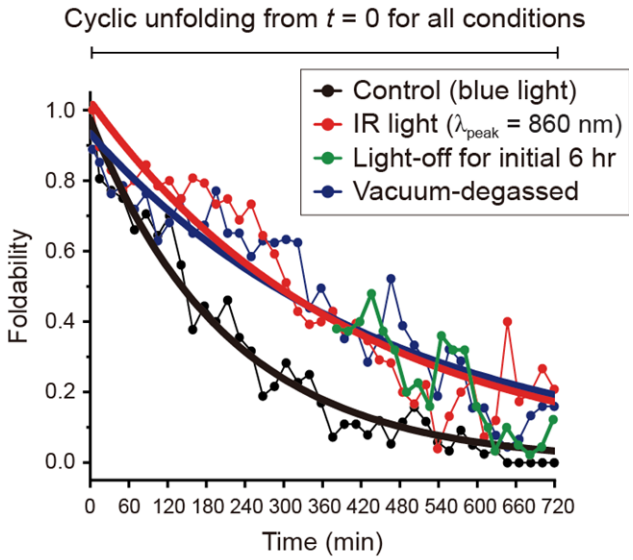


Fig. 1e. Foldability trend over time under different light conditions.

We found that compared to blue light (λ_{peak} = 447 nm, 9.2 mW/cm^2), the decay rate of protein foldability slowed under IR light (λ_{peak} = 860 nm, 26.3 mW/cm^2), similar to what was observed with the vacuum-degassed buffer (with reduced O_2 concentration). We also observed an increase in foldability under no-light condition, *i.e.*, zero light intensity for the initial 6 hr. During the cyclic unfolding experiment, we turned the light off for the initial 6 hr (no data collected during this period due to the absence of light for imaging) and then turned it back on. We found that the foldability level was enhanced under the no-light condition, similar to the IR-light and vacuum-degassed conditions.

The result for the no-light condition was already included in the original manuscript in Fig. 1e. We have now added the new result for the IR condition in the figure panel and a descriptive sentence on page 6, line 5:

"…Under IR light with λ_{peak} = 860 nm, even at higher power, the foldability decay rate also slowed to a similar extent as in the vacuum-degassed buffer (Fig. 1e and Methods)…"

4. Why does oxygen tend to be trapped in hydrophobic cavities? Due to hydrophobic interactions?

Yes, we believe so, based on relevant analyses described in the original manuscript. O_2 is nonpolar, so it is more likely to interact with hydrophobic areas of the protein. The relevant paragraph can now be found on page 7, line 27:

> "The primary O_2-trapping areas (Supplementary Figs. 9 and 10 and Supplementary Tables 2 and 3) are deeply located from the protein surface (Supplementary Fig. 12a,b), and contain a large portion of hydrophobic residues even more than the protein core region (Supplementary Fig. 12c). A hydropathy analysis shows that the O_2-trapping areas also exhibit larger hydrophobicity than nearly all successive sequence blocks with an arbitrary 15-residue window (Supplementary Fig. 12d–f). This analysis indicates that the distant residues in the sequence space would form the hydrophobic O_2 pockets. Additionally, the O_2-trapping times in MBP are comparable to or even longer than the binding times of its natural substrate, maltose (Supplementary Figs. 13–17 and Supplementary Videos 1 and 2). These results suggest that the capture of nonpolar O_2 molecules is mainly mediated by hydrophobic interactions within the cavity-forming areas."

5. Fig. 3f is not understood. The three EPR spectra appear to be similar. Could the difference be highlighted?

To check reproducibility, we purchased fresh TEMP and measured the EPR spectra in triplicate. We observed results similar to those in the original manuscript (Fig. 3f). We have updated the EPR spectra, and the difference between them is now clearly shown.

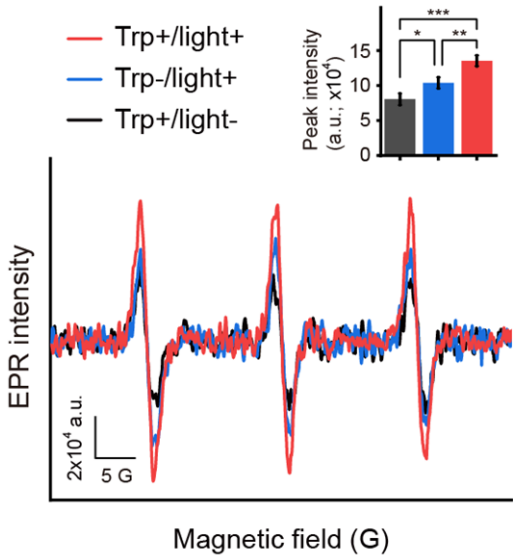


*Fig. 3f. Electron paramagnetic resonance (EPR) measurement using TEMP for $^1O_2$ detection. The EPR spectra are averaged from triplicates for each condition and are expressed in arbitrary unit (a.u.). The inset shows the EPR intensity for each condition (n = 3; mean ± SD). One-way ANOVA with post-hoc Tukey HSD test (*** for p < 0.001, ** for p < 0.01, and * for p < 0.05).*

Reviewer #1

This version of the manuscript is significantly improved over the original version, and the new data included has allowed further clarification of the reactive oxidants involved.

The authors have downplayed the role of singlet oxygen, but this is still postulated as a significant species involved in the oxidation of the detected residues.

We understand your concern. In the previous revised version, we thus downplayed the relevant section and did not emphasize that singlet oxygen ($^1O_2$) is a particularly significant ROS species in residue oxidation. The oxidized residues appear to be affected by various ROS through multiple photochemical pathways, and it is currently unclear which ROS species are more dominant. We discussed this point in the main text as follows:

On page 14, line 17,

> "The oxidized residues of MBP identified in the LC-MS/MS analysis encompass a broad range of amino acid types, including those with aromatic rings and alkyl side chains. This result is likely attributed to various ROS, such as $^1O_2$ and $^{\bullet}OH$, possibly generated through multiple photochemical pathways (spin-flip and type I), though it remains unclear which pathway is more dominant…"

On page 10, line 13,

> "…All our experiments and calculations described in this section collectively support the possibility of blue light-induced generation of various ROS, including $^1O_2$ and $^{\bullet}OH$, near Trp residues through multiple photochemical processes."

This conclusion is however not supported by the additional experimental data provided in the new version of the manuscript, as the presence of D2O does not give rise to any significant enhancement of damage, as would be expected if this species was involved. A number of previous studies on protein oxidation using other sources of 1O2 have shown marked effects of the presence of D2O on markers of oxidation (see, e.g. PMID: 39299525).

There seems to be some misunderstanding. Using the ABDA assay in D_2O, we did not observe protein damage by $^1O_2$. In this assay, ABDA molecules can react with $^1O_2$, which is indicated by a change in absorbance of ABDA at 400 nm. For the tryptophan (Trp) solution specifically, we observed a similar trend in absorbance change in both D_2O and H_2O—a gradual absorbance change of ABDA with Trp, in contrast to the lack of noticeable absorbance changes with the other tested amino acids.

In this regard, this comment does not particularly contradict our results. If D_2O increases the lifetime of $^1O_2$ and significantly affects protein oxidation, Trp would be among the most affected and modified residues. In fact, this point was already noted by this same Reviewer (see comment #2 for our original manuscript). Thus, the results from the ABDA assay—a slightly lower extent of absorbance change of ABDA in D_2O compared to H_2O—may be attributed to a higher rate of oxidative degradation of Trp by $^1O_2$ due to its increased lifetime, resulting in a reduced amount of intact Trp available for $^1O_2$ generation. We have already discussed this point in the previous revised manuscript. We have made slight additional revisions to avoid misinterpretation as follows (with changes underlined).

On page 9, line 1,

> "…For Trp dissolved in H_2O containing ABDA as a $^1O_2$ probe[37,38], we observed a gradual decrease in absorbance of ABDA at 400 nm under blue light irradiation, indicating $^1O_2$-induced ABDA oxidation (Fig. 3e and Supplementary Fig. 19). In contrast, other tested amino acids (aromatic: Phe, charged: Glu, hydrophobic: Ala) did not show noticeable changes in the ABDA absorbance (Fig. 3e and Supplementary Fig. 19). The same trend was also observed in D_2O, which increases the lifetime of $^1O_2$ (ref. [38]), albeit with a slightly lower extent of change for Trp (Fig. 3e). This result may be attributed to a higher rate of oxidative degradation of Trp by $^1O_2$

due to its increased lifetime[22], resulting in a reduced amount of intact Trp available for $^1O_2$ generation…"

Thus the new data provided by the authors does not support the intermediacy of 1O2 to a significant extent, and the corresponding sections of the manuscript should be amended accordingly.

We agree that $^1O_2$ may not be a significant ROS species in residue oxidation. Thus, we discussed this point in the main text. Please refer to our first response above.

The concept that oxidants (such as 1O2) only modify surface residues is not widely accepted (as suggested by the authors – e.g. at lines 50-51 and 411-412), as the authors of the paper indicated above, and also others, have reported that the extent of oxidation does not correlate with the solvent accessible surface areas of the relevant residues.

We apologize for the misunderstanding. Our intent was not to imply that oxidants modify *only* surface residues. Therefore, we have revised the relevant sentences to include the adverb 'primarily' to avoid misinterpretation.

On page 3, line 11,

"…These ROS then freely diffuse within cells or solutions, primarily leading to the oxidation of easily accessible protein surface residues[12-17]…"

On page 14, line 6,

"…This oxidation process stands in sharp contrast to conventional oxidation pathways, which involve the free diffusion of ROS in cell or solutions, primarily leading to the oxidation of easily accessible surface residues of proteins[12-17]."

Many reports show that solvent-accessible surface areas (SASA) correlate with the extent of protein oxidation (PMIDs: 30846556, 35346688, 28470663, 21491861, 14750862, and 12605858). Specifically, in a previous proteomics-based study (PMID: 30846556), a large number of human cell proteins (n = ~3000) were examined, providing strong statistical evidence for this correlation. Additionally, this correlation makes physical sense if you ponder over this question—how could (hypothetical) *perfectly solvent-inaccessible* residues be oxidized? It is physically impossible.

There may be two possible reasons for the lack of a specific correlation in the previous study mentioned by the reviewer (PMID: 39299525). First, the solvent accessibility varies depending on thermal fluctuations in protein conformation. The authors estimated the ratios of SASA based on "static" protein structures derived from AlphaFold. This approach can substantially underestimate these values, as it does not account for "dynamic" protein fluctuations. In contrast, our approach using molecular dynamics (MD) simulations inherently reflects the protein flexibility and motional dynamics. In fact, this point was noted by this same Reviewer (see comment #7 for our original manuscript). Second, in the study mentioned by the Reviewer, the authors used visible light (350–850 nm) to induce photooxidation and measured the extent of protein oxidation. This wavelength range includes the blue light region (~450 nm), and the tested proteins contained Trp residues. Thus, the weaker correlation with solvent accessibility may be "partly" due to contributions from another photochemical mechanisms, such as the O_2-confined photooxidation pathway proposed here.

Based on the discussions above, we hope that the minor text edits will be acceptable.

Minor point:

The authors state that the HPF assay is highly specific for hydroxyl radicals (line 273). This is incorrect. This probe also reacts with a number of other oxidants including peroxynitrite (see PMID: 34050474) and also peroxidase systems. Some of the commercial vendors of this

product (e.g. Thermo Fisher) clearly state this limitation. The authors should note this lack of specificity, as otherwise this (incorrect) concept will be promulgated.
We have revised the main text according to this Reviewer's comment.

1) Achieving 100% specificity should be impossible, especially for ROS, as they are intrinsically highly reactive species. In this regard, specificity is a relative concept—while one may consider a method with 99% specificity to be *sufficiently specific*, others may still view it as *relatively unspecific*. Given this point and the context in which we compared the HPF assay's specificity for •OH with the less specific DHR123 assay, our wording should not pose a serious issue. However, to prevent any potential misunderstanding, we have revised the wording from "highly specific" to "more specific.

On page 9, line 29,

> "…We confirmed Trp-mediated production of type I ROS using two spectrophotometric assays: the less specific DHR123 assay, which provides broad detection coverage of type I ROS, including $O_2^{\bullet-}$, H_2O_2, and •OH (refs. [43-45]), and the more specific HPF assay for •OH, which is approximately 90–360 times more sensitive than for $O_2^{\bullet-}$ and H_2O_2 (ref. [46])…"

2) Although the HPF assay can also respond to peroxynitrite ($ONOO^-$), it is six times more specific to •OH than to $ONOO^-$ (PMID: 12419811). Additionally, $ONOO^-$ is primarily generated in more complex systems like living cells, as in the study referenced by the Reviewer (PMID: 34050474). In cellular environments, nitric oxide (NO) and superoxide (O_2^-) are produced during arginine metabolism and cellular respiration (PMID: 27478531), respectively. These two ROS then react to form $ONOO^-$ (PMID: 27478531). In contrast, our *in vitro* system is very simple, containing only deionized water and an amino acid, with no catalytic or enzymatic reactions. Given the higher specificity of the HPF assay for •OH and the simplicity of our detection system, it is highly likely that the HPF assay results are primarily due to •OH. We have discussed this point in the relevant section as follows.

On page 10, line 6,

> "Although the HPF assay can also respond to peroxynitrite ($ONOO^-$), it is six times more specific to •OH than to $ONOO^-$ (ref. [46]). Additionally, $ONOO^-$ is primarily generated in more complex systems such as living cells[47]. In cellular environments, nitric oxide (NO) and $O_2^{\bullet-}$ are produced during arginine metabolism and cellular respiration[18], respectively. These two ROS then react to form $ONOO^-$ (ref. [18]). In contrast, our *in vitro* system is very simple, containing only deionized water and an amino acid, with no catalytic or enzymatic reactions. Given the higher specificity of the HPF assay for •OH and the simplicity of our detection system, it is highly likely that the HPF assay results are primarily due to •OH..."