Peer Review File

Manuscript Title: A lysine-cysteine redox switch with an NOS bridge regulates enzyme function

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Ref #1

The study by Wensien and coworkers describes the discovery and atomic resolution structure of a previously undescribed intra-protein amino acid crosslink (Lys-Cys, likely, N-O-S) that upon reduction increases kcat 30-fold and decreases KM 2-fold of a transaldolase, together leading to a significant increase in activity.

There are three important observations in this study:
1. The observation of a new covalent crosslink which when reduced results in higher enzyme activity in NgTAL.
2. The crosslink is found in transaldolase from N. gonorrhoeae, providing a possible link to disease and drug design.
3. Mining of the PDB identifies other proteins that may contain this crosslink.

The methodology is sound and the data quality and refinement statistics in the crystallographic analysis is solid. Complementary biochemical and mutational studies are provided.
The text is short, clear and easy to follow, in my mind the wording is unnecessarily ostentatious in places "to resolve this apparent ‚riddle wrapped in a conundrum inside an enigma" but this may be personal preference.
The figures are clear and accessible.

Main issues:

While the activation by reduction is clearly shown, the authors do not show it is a "redox switch" or employed as a regulatory mechanism, only that the protein as prepared can be activated by reduction. An alternative (less exciting) interpretation of the data is that the protein as prepared has been adventitiously oxidized to a less active state. In my mind, to be able to conclude that this is indeed a regulatory mechanism, further support is needed.
First, it needs to be shown that the modification is reversible so that the protein can be re-inactivated by oxidation as suggested in the conclusions. An experiment showing reoxidation and concomitant inactivation would significantly strengthen the study.
Second, some support that it is indeed used as a regulatory mechanism in vivo should be provided, while this may not be trivial to obtain, one could at least study homologous protein sequences to see if the two residues involved are conserved. Potentially, in vivo studies using knock-ins of protein variants (e.g. always active or inactive) to observe phenotypic behavior could be used. I fully understand that it may be difficult to prove in vivo relevance but it appears required to support the claims made, e.g. in the title "An allosteric lysine-cysteine redox switch with a covalent N-O-S linkage regulates enzyme function".

The discussion regarding the mechanism of formation of the crosslink is very speculative and it appears to me that before such discussions, more should be established regarding the prerequisites for the reaction. The authors speculate that molecular oxygen (putatively also directly observed in the reduced structure) is the oxidant. Before mechanistic discussions it appears necessary to establish that this is the case. This could be achieved either by showing oxygen-dependent reoxidation (see above) or, if this is not possible (e.g. if additional unknown factors are required) at least the involvement of oxygen could be established by anaerobic expression and purification which should render an active non-crosslinked protein.

The experiments above would also strengthen the assignment of the bridging species as an oxygen atom. While the crystallographic analysis based on the electron density makes this assignment plausible, even at atomic resolution element identification in this way cannot be viewed as fully conclusive. In this perspective it is unfortunate that the Mass-spec analysis was not able to provide the mass of the crosslinked peptides.

Finally, the identification of this crosslink in other proteins in the PDB is indeed very interesting and strengthens the potential general importance of this discovery. However, these are identified purely by analyzing the (sometimes low resolution) electron density of already deposited structures. More importantly, there is no data to support that these modifications influence the function, or that they are not just the result of adventitious reactions when a Cys happens to be close to a lysine sidechain under certain conditions (as also discussed above) mining the >150 000 structures in the PDB will inevitably result in the discovery of many more or less biologically relevant features. For these reasons, statements such as "the NOS link is not a unique feature of NgTAL but constitutes a widespread chemical entity in proteins with dedicated roles in protein function and stability" would require additional experimental support.

This is an exciting study and the discovery of a new covalent crosslink is notable and I congratulate the authors for this. If this is indeed a widespread regulatory redox switch is certainly very important but, in my mind, additional experiments are required to support the claims made.

Minor:
Please draw out the location of Lys8 in fig 2a.

Ref #2

This manuscript describes the discovery in an enzyme of a reversible covalent crosslink between a cysteine and a lysine, bridged by an oxygen atom. The authors show that formation of this bridge decreases activity of the enzyme (transaldolase from Neisseria gonorrhoeae). Reduction in turn restores activity. Mutagenesis and computational analyses support a proposed mechanism for formation of the linkage. Lastly, and very importantly, the authors show that an oxygen-bridged cysteine-lysine pair exists in a variety of other proteins but was previously missed.

A novel chemistry for protein crosslinking that has functional significance is a fundamental discovery, so this reviewer was excited to read and review the results. Overall the data look convincing, but many improvements to the manuscript can be made. Because the discovery is really quite remarkable, the paper deserves additional effort to perfect the presentation. The interpretation of the circular dichroism data needs some revision, there are a few illogical statements in the text, some of the extended data figures are illegible, and the whole manuscript is unnecessarily wordy. The various scientific and stylistic issues are listed below in the order in which they appear in the text.

p. 2 abstract: "protein crystallography of the protein"-- remove the first "protein"
p. 2: it doesn't quite make sense to say that the crosslink was neglected "due to limited resolution in protein structure determination." The authors identified the linkage in other structures, indicating that these structures were determined to sufficient resolution. The problem, at least in some cases, was that people passed over the evidence because they weren't looking.
p. 2 introduction: should be "three-dimensional structures" (plural) since each protein has its own structure. Also, what does "defined" mean here? Better would be "are formed and maintained."
p. 2-3: can shorten to: "In addition to peptide bonds, only a few additional covalent bonds between amino acids contribute to…" This phrasing is also more accurate, since of course many other covalent bonds (the backbone N-C, C-C, and all covalent bonds in the side chain) also contribute to protein structure. But these others are not BETWEEN amino acids.
p. 3: The last sentence of the introdution is not completely clear. The second-to-last sentence

described the reversibility of disulfides, but the last sentence seems to include the possibility of a larger variety of cysteine modifications and not just disulfides. To avoid confusion, the authors could perhaps say: "In addition to disulfides, a variety of other oxidized cysteine species serve as molecular sensors…"
p. 3 results: No essential information would be lost by shortening to: "Transaldolase (TAL) is a key enzyme in the pentose phosphate pathway. The Neisseria gonorrhoeae TAL ortholog (NgTAL) is a potential drug target for fighting gonorrhea, the sexually transmitted disease caused by this pathogen. When produced recombinantly, NgTAL showed almost no enzymatic activity. We hypothesized that the enzyme could be subject to activation by a redox event involving one or more of its three cysteines: Cys38, Cys87, and Cys90."
p. 3-4: "supporting our initial hypothesis of a redox activation" should be removed, because the sentence is too long and the "In fact" at the beginning already indicates that the hypothesis was correct.
p. 4: There is no reason to lead the reader astray by mentioning "a putative disulfide-based redox switch." The authors could instead write: "… is not altered by reduction, but differences were seen between the oxidized and reduced states in thermal unfolding." However, see the next comment.
p. 14 Figure 2: It is not clear how the authors measure cooperativity in the thermal unfolding in panel D. If this is a quantitative measure, the method for calculating it should be given. To this reviewer, the slopes at the midpoint of the transitions look similar. The shapes of the folded baselines do look a bit different, but what does that mean exactly? This reviewer assumes that the differences in the folded and unfolded baseline signals between oxidized and reduced states in the melt are due to a protein concentration issue in this experiment, since panel C shows identical signals at 222 nm. At what temperature was the data in panel C collected? Why are the data in panel D not presented as molar (mean residue) ellipticity as in panel C? In general, the analysis (or at least the explanation) of the CD data should be improved.
p. 4: After correcting and condensing the description of the differences in the thermal melts, the authors should start a new paragraph without mentioning disulfides: "To determine which of the cysteines in NgTAL is involved in redox regulation, we constructed variants lacking each of the three cysteines. We predicted that substitution…" Disulfides are irrelevant to the story at this point.
p. 4: "We thus hypothesized that Cys38 might form an intermolecular disulfide bridge…" Here it is perfectly OK to mention disulfides, because now the hypothesis is explicit and not confusing/misleading.
p. 4: delete "seemingly viable" and change "had been" to "was" or "has been."
p. 4: delete "using a set of different protein concentrations." This information can be provided in the methods.
p. 4: Should be "The enzyme was predominantly a monomer under all conditions tested, falsifying our hypothesis of a redox switch involving a change in quaternary structure." (The authors should be consistent about using the past tense to describe results of experiments, and it is sufficient to mention the minor dimeric population in the figure legend as is already done.)
p. 5: There is absolutely no need for riddles, conundrums, and enigmas! Or for misquoting Churchill. This section should read: "To further investigate the basis for redox activation, we crystallized NgTAL under oxidizing and reducing conditions. We determined structures of both states at true atomic resolution…"
p. 5: A quick search on Chemspider reveals that an N-O-S substructure is present in many molecules. But indeed they do seem to have higher oxidation states for the sulfur (but not always the same one as hydroxylamine-O-sulfonic acid). It also seems not quite right to compare the number of oxygens bound to the sulfur in HOSA with the number in the NgTAL linkage, since in cysteine the sulfur is bonded to a carbon, which is absent in HOSA. There are examples of the N-O-S substructure that are actually N-O-S-C, and in these cases the sulfur is typically bonded to either 2 or 3 oxygens.
p. 5: "(no other oxidants were present in solution)" can be omitted.
p. 6: It is not quite clear which side chains are close enough to constitute the hydrophobic patch in which the O2 is found. Ile14 is labeled behind the O2 and may be close enough to be relevant, but Leu4 and Val37 look too far away. Can the authors specify, perhaps in the figure legend, which

residues make up the patch?
p. 6: There is no reason one should be confident about a suggestion, only about a conclusion. Perhaps rephrase: "… it is difficult at this point to determine the chemical mechanism for formation of the N-O-S linkage, especially as there is no precedent…"
p. 6: The authors should be consistent and use either N-O-S or NOS, but not both
p. 6: should be a comma after "do react with amines"
p. 6: rephrase: "Electronic structure calculations were carried out to compare…" ("in order to" means the same as just "to")
p. 6: omit "safely" in "we can safely rule out"
p. 7: The paragraph on the electronic structure calculations seems to end without any clear suggestion for the mechanism. In contrast, the legend of extended Figure 3 does seem to make some suggestions. Even if the authors are not 100% sure, they can still improve the clarity of this paragraph by explaining why they disfavor various mechanisms outlined in the previous paragraph and which remain(s) as possible viable mechanism(s). Some of this information is present in the paragraph already, but not as coherently as could be.
p. 7: shorten: "To understand the structural basis for the decrease in NgTAL activity upon formation of the N-O-S linkage, we superposed the oxidized and reduced structures (Figure 4)."
p. 7: should be "strand-like structure" or "extended, strand-like structure" rather than "sheet-like structure."
p. 7: should be "It is known for this enzyme and for many other enzyme classes… critical for catalysis, with small…"
p. 7: This reviewer found the comparison to GTPases distracting rather than helpful. The authors may want to consider deleting this comparison and ending the paragraph with the previous sentence.
p. 7: The sentence "We next asked…" is problematic, since formation of the NOS linkage causes DE-activation of the enzyme, not activation.
p. 7-8: Simplify to "We thus generated Lys8Ala and Cys38Ser variants, which cannot form the crosslink. Lys8Ala showed a ~7 °C difference in melting temperature between the oxidized and reduced states, whereas Cys38Ser showed almost identical melting temperatures in the two states. This observation suggests that…"
p. 8: Perhaps start with: "The chemical identity of the N-O-S link appears well established on the basis of X-ray crystallography, but we tested whether this linkage could also be detected by mass spectrometry (MS). MS is routinely used for detection of disulfide bonds (28,29), and detection of N-O-S bonds would obviate the need for ultra-high resolution X-ray crystallography."
p. 8: A lack of evidence is not evidence, so perhaps rephrase: "We were, however, unable to detect the crosslink between the two peptides bearing Lys8 and Cys38, and instead Cys38 was found predominantly in the sulfinic acid state (Extended Data Figure 5). It thus appears that the covalent NOS linkage is hydrolyzed under the acidic conditions used for sample preparation. The MS data are nevertheless informative, as they provide further support that the bridging atom is indeed oxygen. No modifications of Lys8 could be detected either in the oxidized or reduced enzyme, and Cys38 was detected as the unmodified thiol under reducing conditions."
p. 8: shorten and modify: "The chemical instability of the NOS crosslink during sample processing, coupled with the absence of this species in the modifications searched during MS data analysis, explains why it has been missed in proteomics analyses so far." The authors may be able to improve upon the suggested phrasing, but the point is that even if the crosslink had been stable, people still would have missed it in proteomics MS experiments unless they had known to specifically search for it.
p. 9: Should be: "We next asked whether the Cys-Lys NOS link exists in other proteins. We searched the protein structure database (www.rcsb.org) for juxtaposed lysine and cysteine side chains with unexplained…"
p. 9: Remove "Obviously" in "Obviously, one requires…"
p. 9: "we could identify numerous proteins from all domains of life"-- six proteins are shown in the supplementary figure. Were more than these identified? If not, perhaps delete "numerous," which suggests at least tens if not hundreds (considering the size of the PDB).
p. 9: change "most" to "many" in "most proteins are purified…" unless the authors can provide

numbers regarding how many PDB entries were produced from crystals grown in the presence of reducing agents. Many secreted, cell surface, or organelle-localized proteins are disulfide bonded, and crystallographers working on those would not add reductant during crystallization.
p. 9: It is strange to read, "In conclusion…" and then right afterwards see a section headed "Conclusions."
p. 9: Grammatically, one showcases something. One does not showcase that something. Also, the authors did not demonstrate in the other systems that the linkage has a role in either function or stability. Simplify to: "Our analysis shows that the NOS link is not unique to NgTAL and may contribute to function and stability in a diverse set of proteins."
p. 9, Conclusions: "eliciting" is used incorrectly here. Increasing is the correct word.
p. 10: Again, it is true that lability causes a problem for MS, but it is also true that people need to know what to look for to find modifications by MS (and other techniques). That is why this paper is so important-- it reveals a completely new and unexpected thing to look for.
p. 13, Figure 1A: What is the meaning of the double arrows going from thiol and sulfenic acid (third row in panel a) to intermolecular disulfide (second row in panel a)? Yes, thiols can be oxidized to disulfides, but this is a redox reaction, and unlike the other processes shown in the third row, no other redox species are shown to be involved. Also, these arrows are confusing because they don't show where the red-sphere species comes from.
p. 16, Figure 3: The distances are in very small type in panels c and d. There is a general problem in all the figures of the paper that the font sizes vary a lot, and not always does the size reflect the importance of the information.

Extended data:
p. 4: The yellow on a gray background is very difficult to see. This reviewer asked some much younger people (with better eyesight), and they agreed.
p. 7: The size of the text is too small in the table, and the chromatography peaks and labels are miniscule.

Ref #3

This manuscript reports a subatomic resolution X-ray crystal structure of transaldolase from a human pathogen N. gonorrhoeae, and the novel discovery of an unprecedented N-O-S linkage in the protein structure. Based on the melting behavior and the kinetic assays in the presence or absence of reducing reagents, the authors propose that such an N-O-S linkage is part of the previously unknown redox switch in the cell in response to reactive oxygen species. By reanalyzing the electron density of deposited crystal structures in the PDB, the authors further propose that this mechanism is not uncommon in biology but has been largely overlooked in the past. The chemical mechanism of the N-O-S linkage formation is proposed based on the presence of dioxygen as well as from relative free energies calculations.

Overall, the structure of NgTAL is beautifully resolved at subatomic resolution and shows unique features compared to the previously determined NgTAL structure (PDB 3CLM, 1.14 Å). The proposed assignment of the linkage atom seems reasonable due to the subatomic resolution. However, whether this species is biologically relevant or an artifact remains questionable. The reasons for this are described below.

First, no additional evidence (besides the crystal structure) appears to support the existence of the NOS linkage, raising the question of whether the observation is due to radiation damage. To rule this out, the authors need a solid MS analysis, at a minimum, to compare the protein crystal samples before and after X-ray data collection. It seems that the lack of an NOS linkage in the LC-

MS analysis was rationalized due to the acidic condition when running LC-MS. Many other current MS techniques could overcome this issue or simply a change of buffer/column. If this NOS linkage exists in the crystal structures that authors use to generalize the wide distribution of NOS linkage across all kingdom, some of the ascribed enzymes were likely resolved under acidic conditions, such as PDB 5Y72 and PDB 5YZP, and, as such, would not be expected to retail the proposed NOS crosslink.

Second, the activity observed in the kinetic assay containing DTT is not enough to support biological relevance for the NOS structure. As the authors mention in the MS analysis, modifications have been observed on the cysteines, suggesting that the purified enzyme in the absence of reducing reagent has already undergone oxidative damage during sample preparation. It is not surprising that low catalytic activity is observed for this protein sample. Additionally, since the kinetic assay is a coupled assay using triosephosphate isomerase (TIM) and sn-glycerol-3-phosphate:NAD+ 2-oxidoreductase (G3PDH), a control experiment indicating whether DTT impacts the coupling reagents is lacking in the manuscript.

A minor point, the crosslink between cysteine and other residues, such as Trp, Tyr, Arg and Glu, is not unique to galactose oxidase. These cysteine crosslinks also exist in enzymes such as cysteine dioxygenase (McCoy, J. G., et al. 2006 Proc Natl Acad Sci, 103, 3084-3089.), lysine ε-oxidase (Okazaki, S. et al. 2013 J Biochem, 154, 233-236.) and quinohemoprotein amine dehydrogenases (Datta, S., et al. 2001, Proc Natl Acad Sci 98, 14268-14273). It is even more diverse if small proteins and peptides are included, such as sactipeptides (Balty, C. et al. 2019 J Biol Chem, 294, 14512-14525, & Kawulka, K. E., et al. 2004 Biochemistry, 43, 3385-3395.) and thiopeptides (Kelly, W. L. et al. 2009 J Am Chem Soc 131, 4327-4334.). The statement in the abstract that "native crosslinks with neighboring amino acids other than cysteines have not been demonstrated for proteins with one notable exception" is not accurate.

Author Rebuttals to Initial Comments:

Reviewer 1

1) While the activation by reduction is clearly shown, the authors do not show it is a "redox switch" or employed as a regulatory mechanism, only that the protein as prepared can be activated by reduction. An alternative (less exciting) interpretation of the data is that the protein as prepared has been adventitiously oxidized to a less active state. In my mind, to be able to conclude that this is indeed a regulatory mechanism, further support is needed. First, it needs to be shown that the modification is reversible so that the protein can be re-inactivated by oxidation as suggested in the conclusions. An experiment showing reoxidation and concomitant inactivation would significantly strengthen the study.

Authors: The redox switch is indeed reversible. The reduced enzyme re-forms the NOS bridge, we could confirm this by X-ray crystallographic structure analysis.

We added at p.3/4: "This redox activation is reversible, the reduced enzyme slowly converts into the oxidized form over a couple of days as confirmed by X-ray crystallography (see below)."

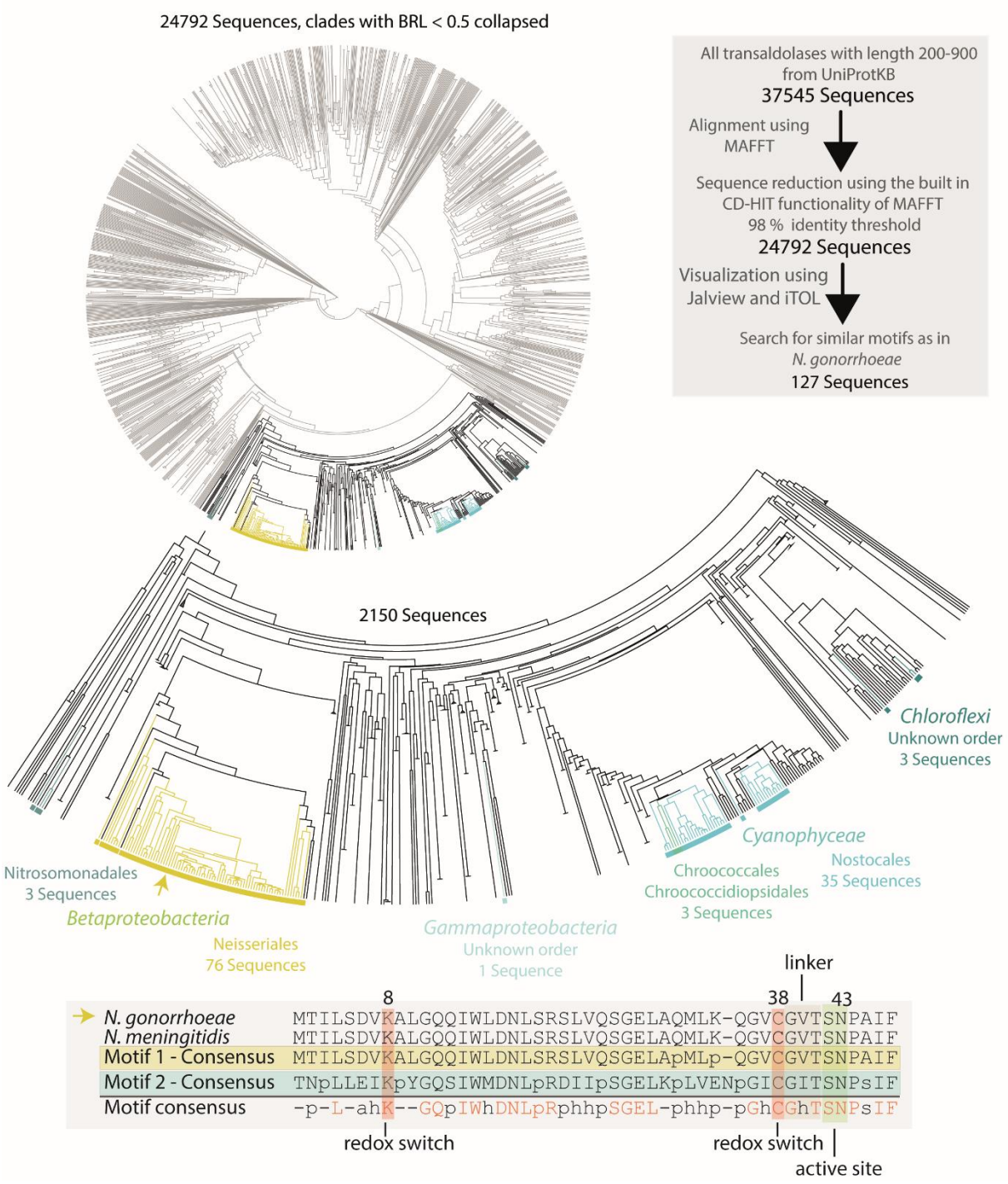
2) Second, some support that it is indeed used as a regulatory mechanism in vivo should be provided, while this may not be trivial to obtain, one could at least study homologous protein sequences to see if the two residues involved are conserved. Potentially, in vivo studies using knock-ins of protein variants (e.g. always active or inactive) to observe phenotypic behavior could be used. I fully understand that it may be difficult to prove in vivo relevance but it appears required to support the claims made, e.g. in the title “An allosteric lysine-cysteine redox switch with a covalent N-O-S linkage regulates enzyme function”.

Authors: We agree with the reviewer. It is, however, extremely difficult to obtain relevant *in vivo* data in a reasonable time frame as that would require to work with *Neisseria* strains, which are pathogenic and not easy to genetically manipulate. But we conducted a phylogenetic and sequence analyses and could identify that the NOS-bridge forming residues are conserved in *Neisseriaceae* and other clades. As proof-of-concept, we studied the transaldolase ortholog from *Neisseria meningitidis*, which according to sequence conservation should have the lysine-cysteine redox switch and find it indeed to be redox-activatable akin to the title enzyme. We added this information to the manuscript (in main text and new ED Figures 6 & 7).

We added at p. 9:

Conservation of NOS bridges in transaldolases

We next asked whether the observed NOS bridge is a unique finding for transaldolase from *Neisseria gonorrhoeae* or potentially conserved in related orthologs. One would argue that a potential redox regulation mechanism with *in vivo* relevance would be very likely conserved and not be a unique feature of a single protein. Our genetic analyses revealed the NOS bridge-forming residues Lys8 and Cys38 are highly conserved in *Neisseriaceae* but also in other clades as e.g. in *Cyanophyceae* (ED Fig. 6). As proof-of-concept, we studied the transaldolase from *Neisseria meningitidis* (*Nm*TAL) the main cause of bacterial meningitis and septicemia in children and young adults. This enzyme is highly similar to *Ng*TAL and also contains the NOS bridge forming lysine and a cysteine at the equivalent positions. Our functional and structural analyses of *Nm*TAL could indeed reveal that the enzyme is subject to redox activation (ED Fig. 7, ED Table 1). Besides the impact on enzymatic activity, we noted that the oxidized enzyme with the NOS bridge likely being present exhibits a remarkable thermal stability and is not completely unfolding. The high degree of conservation of NOS bridge redox switches in *Neisseriaceae* suggests that its formation is related to ROS stress response as these organisms are all human pathogens and encounter oxidative stress as a host defence mechanism [31,32]. Formation of the NOS bridge in the transaldolases would change the metabolic flux through the pentose phosphate pathway and thus impact ribose production for nucleic acid biosynthesis. One might speculate that this metabolic adaption under oxidative stress would be beneficial to allow DNA repair with e.g. excission of oxidized bases rather than an unreduced ribose production for DNA synthesis.



Extended Data Figure 6. Phylogenetic analyses and sequence conservation of the Lys-Cys redox switch in the transaldolase protein family using *Ng*TAL as reference. Two related consensus motifs were identified that contain the lysine and cysteine residues of the redox switch, the active site serine and asparagine residues required for catalytic activation of the Schiff-base forming lysine and the linker region connecting the redox switch with the active site. Note that the identified motifs are highly conserved in *Betaproteobacteria*, in particular *Neisseriales* (motif 1), and in *Cyanophycea* (motif 2). The transaldolase from *N. menigitidis*, a leading cause of meningitis and septicemia in children, is highly similar (95% identity) to the one of *Ng*TAL and also contains motif 1. Structural and functional analyses of transaldolase from *N. menigitidis* could confirm the existence of a redox switch (see ED Fig. 7).

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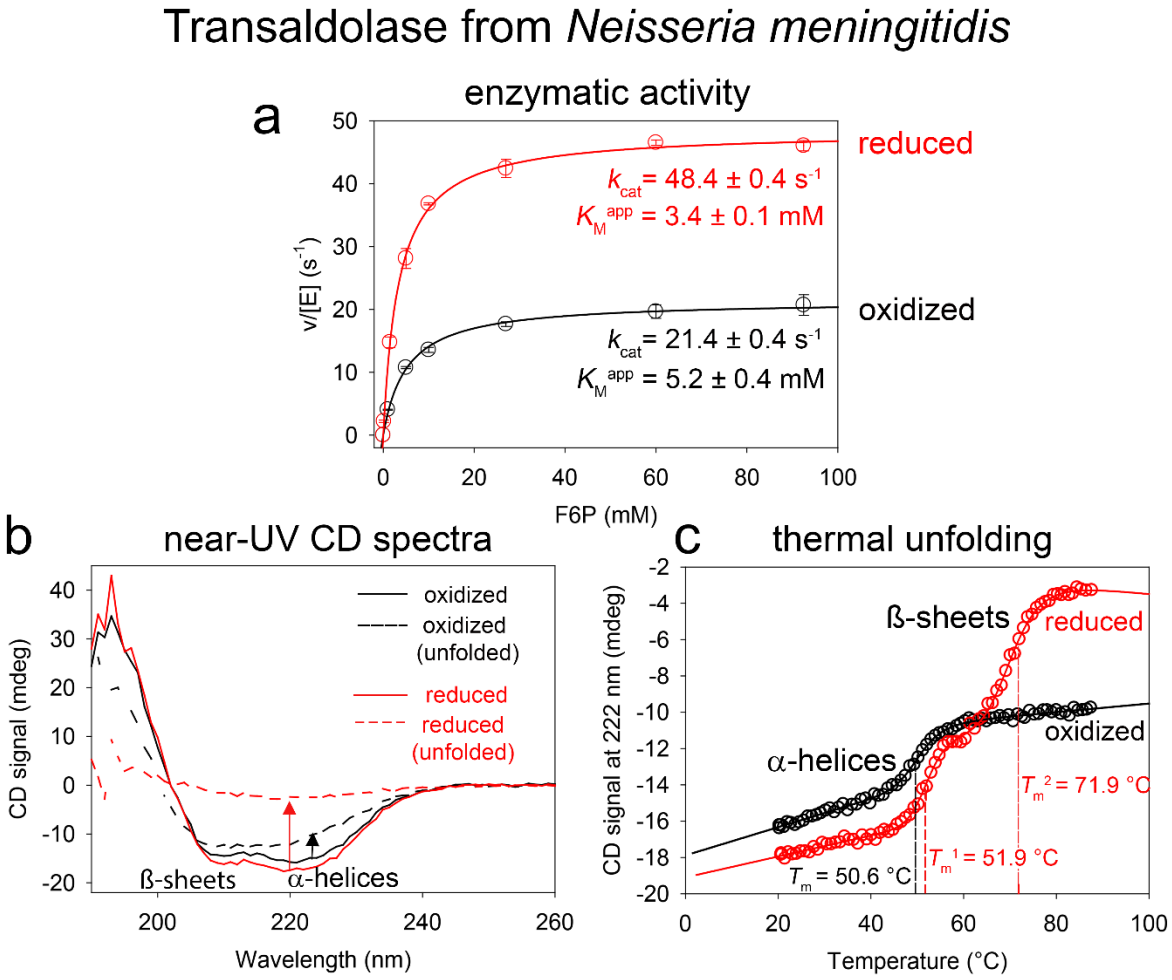

Extended Data Figure 7. Structural and functional analyses of transaldolase from *Neisseria meningitidis* (*Nm*TAL) indicate the presence of a redox switch. (**a**) Steady-state kinetic analysis of enzymatic activity of *Nm*TAL in the oxidized (black) and reduced (red) state. Note the multi-fold increase of k_{cat} and concomitant decrease of substrate K_M upon reduction. (**b**) Near-UV circular dichroism (CD) spectra of *Nm*TAL in the oxidized (black) and reduced (red) state showing both the natively folded states (solid lines) as well as the ones after thermal unfolding (dashed lines). Note that the reduced enzyme completely unfolds and does not contain residual secondary structure. In contrast, the oxidized enzyme exhibits only partial unfolding of mostly helical elements and contains thermally stable ß-sheet structures. (**c**) Thermal unfolding of *Nm*TAL in the oxidized (black) and reduced (red) state monitored by near-UV CD spectroscopy at 222 nm. While the oxidized enzyme displays a mono-phasic unfolding with a melting temperature of 50.6 °C corresponding to unfolding of the α-helices (see panel b), the reduced enzyme shows a bi-phasic unfolding with melting temperatures of 51.9 °C (first transition) and 71.9 °C (second transition). This observation suggests that the putative NOS bridge in the oxidized state of *Nm*TAL specifically stabilizes the interior ß-sheet structure of the TIM barrel in agreement with the position of switch residue Cys38.

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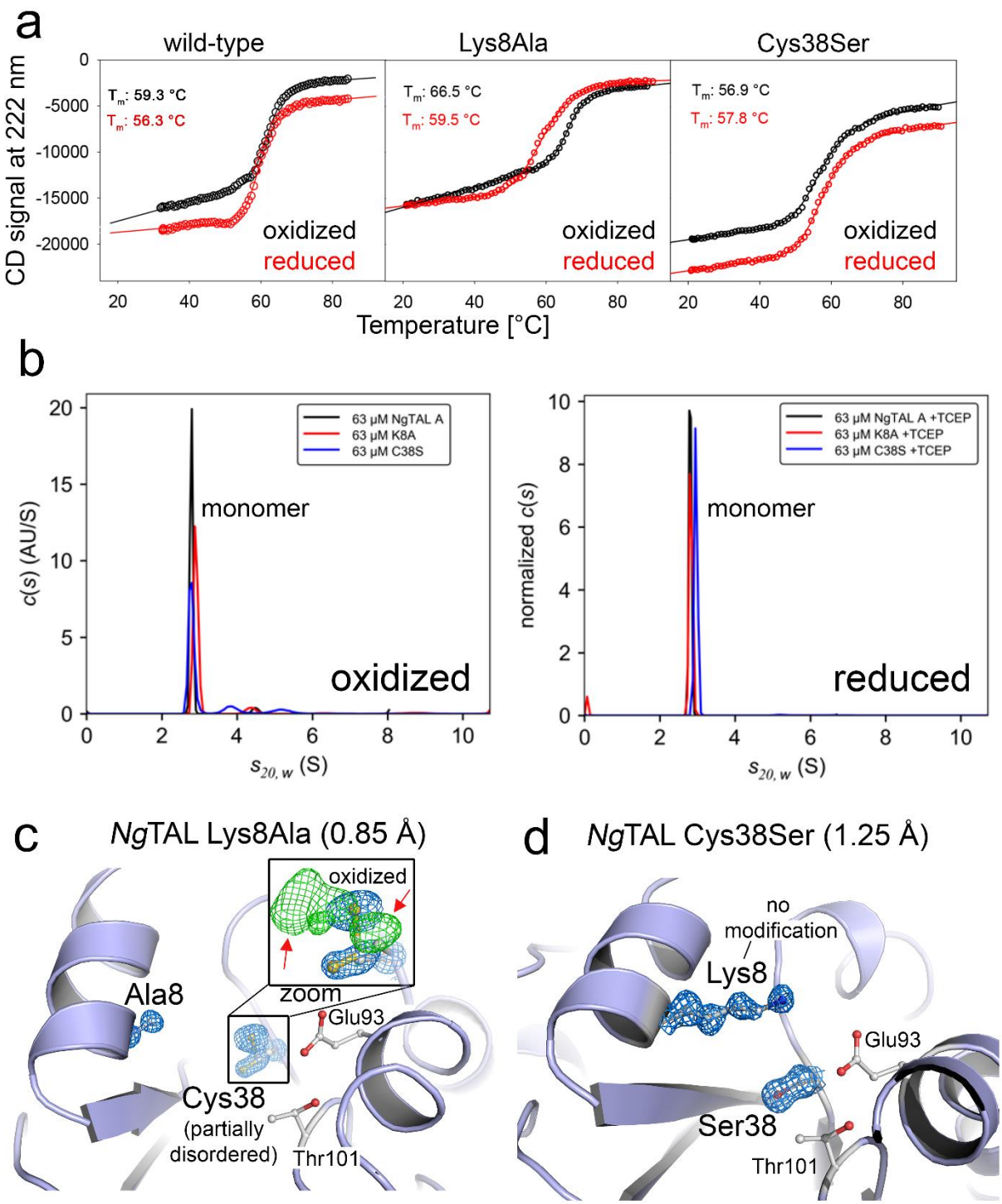
3) The discussion regarding the mechanism of formation of the crosslink is very speculative and it appears to me that before such discussions, more should be established regarding the prerequisites for the reaction.

Authors: We obtained novel insights by both quantum chemical calculations as well as experiments on mutant proteins with substitutions of the NOS-bridge forming residues. These data collectively show that the formation of the NOS bridge results from an initial oxidation of the cysteine side chain (Cys38). We feel that we should provide at least a mechanistic hypothesis to test whether there is a viable chemical route.

We added at p.5:

To define the chemical basis of NOS bridge formation, we studied the individual reactivities of Cys38 and Lys8 in the absence of the corresponding NOS bridge partner. To this end, we analysed structure and function of variants Lys8Ala (Cys38 of NOS bridge present) and Cys38Ser (Lys8 of NOS bridge present) (ED Fig. 2, ED Table 1). While variant Cys38Ser is not redox-activatable and shows identical melting temperatures of unfolding under oxidizing and reducing conditions, variant Lys8Ala is still subject to redox activation and exhibits altered melting temperatures upon redox activation. This suggests that Cys38 in variant Lys8Ala is subject to a chemical modification, most likely an oxidation, while Lys8 in variant Cys38Ser is likely not modified. To test this hypothesis we determined the X-ray crystal structures of both variants at 0.85 Å (Lys8Ala) and 1.25 Å (Cys38Ser) resolution, respectively (ED Fig. 2c, d & ED Table 2). The structural data support our notion that Cys38 in variant Lys8Ala is oxidized, while Lys8 in Cys38Ser is chemically unmodified. These observations bolster our claim that the Cys38-Lys8 crosslink results from an oxidation reaction.



Extended Data Figure 2. Thermal unfolding, analytical ultracentrifugation and X-ray structures of *Ng*TAL variants Lys8Ala and Cys38Ser. (**a**) Thermal unfolding of *Ng*TAL wild-type and variants Lys8Ala and Cys38Ser under oxidizing and reducing conditions as monitored by far-UV CD spectroscopy at 222 nm. Note the different unfolding temperatures for the oxidized and reduced states in case of the wild-type enzyme and variant Lys8Ala, while variant Cys38Ser does not exhibit this feature. This suggests an oxidation of Cys38 in variant Lys8Ala despite the absence of Lys8. (**b**) Analytical ultracentrifugation analyses of *Ng*TAL wild-type and variants in the oxidized and reduced state evidences the predominant formation of the monomeric form in all cases tested. Under oxidizing conditions and high protein concentrations, a small fraction of higher oligomers is observed presumably resulting from incorrectly linked monomers. (**c**) X-ray crystallographic structure of *Ng*TAL variant Lys8Ala showing the allosteric

redox switch site with residues Ala8 (mutation site), Cys38, Glu93 and Thr101. For residues Ala8 and Cys38, the corresponding 2mFo-DFc electron density maps are shown in blue at a contour level of 2σ. Inset: Peaks in the mFo-DFc difference electron density map (in green, contour level +3σ) around the sulfur atom of residue Cys38 suggest the latter to be oxidized. Owing to the structural flexibility of Cys38, the discrete oxidation state (mono-oxidized and/or di-oxidized) cannot be unambiguously assigned. That notwithstanding, this observation supports our proposed mechanism of an initial cysteine oxidation en route to NOS bridge formation. (**d**) X-ray crystallographic structure of *Ng*TAL variant Cys38Ser showing the allosteric redox switch site with residues Lys8, Ser38 (mutation site), Glu93 and Thr101. For residues Lys8 and Ser38, the corresponding 2mFo-DFc electron density maps are shown in blue at a contour level of 1.5σ. Note that Lys8 is chemically unmodified thus ruling out that the covalent linkage between Lys8 and Cys38 seen in the wild-type enzyme results from addition of CO_2 or formaldehyde thus potentially establishing an N-C-S linkage [18].

We have also rewritten the section about the mechanism and updated ED Fig. 3 & 4:

p.6/7

Mechanism of NOS bridge formation

Despite the unambiguous structural information, it is difficult at this point to determine the chemical mechanism for formation of the NOS linkage, especially as there is no precedent for this reaction in organic or protein chemistry. In principle, oxidized cysteines do react with amines, but the nucleophilic nitrogen would rather attack the sulfur atom than a sulfur-bound oxygen to afford sulfonamides or sulfinamides [21]. We envisage different alternative mechanisms for the formation of the NOS bridge. First, the cysteine thiolate could react with dioxygen (or other ROS) to yield a thio-(hydro)peroxy species that is in turn attacked by the ε-amino group of the neighboring Lys8 (ED Fig. 3a). This reaction would directly afford the covalent NOS bridge with concomitant water release facilitated by synchronized proton transfer from the attacking amine onto the water leaving group. In an alternative scenario (ED Fig. 3b), oxygen/ROS might oxidize both the cysteine thiol as well as the lysine amine forming sulfenic acid and hydroxylamine/amine oxide (either in a concerted reaction with a transient thio-(hydro)peroxy species, or, alternatively, in independent reactions). The hydroxylamine/amine oxide is a potent *O*-nucleophile [22] that would have sufficient reactivity to covalently attack an oxidized cysteine. Formation of the NOS bridge would then occur simultaneoulsy with water elimination. In a third mechanism (ED Fig. 3c), the lysine amine would first attack the S atom of the previously oxidized cysteine (either sulfenic or sulfinic acid) followed by sigmatropic [1,2] rearrangement driven by orbital steering and stereoelectronic control. For this to happen, the enzyme would need to destabilize the rather stable N-S bond of the presumed sulfinamide and steer the orbitals to promote an [1,2] rearrangement by e.g. exerting strain [23,24].

Electronic structure calculations were carried out to compare the different mechanistic hypotheses. These were performed on the basis of a cluster model, including Lys8, Cys38 and Glu93, together with four explicit water molecules to retain a hydrogen bond network close to the reaction site. A schematics of the computed mechanisms and free energies is shown in ED Fig. 4. We started our investigations with the thio-(hydro)peroxy species. We identified a transition state for O-O bond breakage and concerted oxidation of Lys8. This step has an activation barrier of 12.2 kcal/mol, leading to the formation of sulfenic acid and the oxidized lysine. We observe that several different protonation states are accessible at this point, with the neighboring waters mediating proton transfer along the residues. We argue that further oxidation of the Cys38 to sulfinic acid is not viable on the way to form the NOS bridge, as sulfinic acid is far too stable (41.4 kcal/mol lower in energy relative to the NOS linkage) effectively trapping the reaction. A potential

sigmatropic [1,2] rearrangement should also be excluded as any pathway involving the sulfinamide intermediate would result in barriers in excess of 35 kcal/mol. Considering the stability and the cysteine oxidation state in sulfinamide and in sulfinic acid, the mechanism for NOS bridge formation must necessarily involve a lower oxidation state (e.g., sulfenic acid). At this point the quantum chemical calculations favor mechanism b (see ED Fig. 3, 4).

4.) The authors speculate that molecular oxygen (putatively also directly observed in the reduced structure) is the oxidant. Before mechanistic discussions it appears necessary to establish that this is the case.

Authors: See answer to point 3 and the novel results on variant Lys8Ala, where Cys38 is found to be oxidized. As there was no other oxidant in solution than O_2, we are convinced that NOS formation in vitro results from oxidation by O_2. Under in vivo conditions, other ROS might be relevant, too (hydrogen peroxide, superoxide anion, peroxide etc). We have started to systematically analyse the reactivity of the redox switch with different reductants and oxidants but would like to address this point in a separate publication as this goes beyond the scope of the current manuscript.

4) While the crystallographic analysis based on the electron density makes this assignment plausible, even at atomic resolution element, identification in this way cannot be viewed as fully conclusive.

Authors: We kindly disagree here. At the obtained resolution of 0.96 Å, the element type can assigned with high certainty by X-ray crystallography. We did – as outlined in the ms – competitive refinements with different bridging atoms. A model with a bridging oxygen was the only one, where no unexplained difference electron density remained. The assignement was further bolstered by the observation of the oxidized Cys in the Lys8Ala crystal structure and by MS.

5) Finally, the identification of this crosslink in other proteins in the PDB is indeed very interesting and strengthens the potential general importance of this discovery. However, these are identified purely by analyzing the (sometimes low resolution) electron density of already deposited structures. More importantly, there is no data to support that these modifications influence the function, or that they are not just the result of adventitious reactions when a Cys happens to be close to a lysine sidechain under certain conditions (as also discussed above) mining the >150 000 structures in the PDB will inevitably result in the discovery of many more or less biologically relevant features.

Authors: We have systematically analysed the whole pdb and detect the NOS bridge in many protein of all domains of life. We can only present the tip of the iceberg here as the structural findings and biological implications of this analysis go beyond the scope of the manuscript. Intriguingly, the NOS bridge is often found at functional or regulatory hotspots and it can be anticipated that the redox switch regulates biological function. We believe that the discovery of the NOS bridge is an important finding. We have picked out 3 key examples for the current manuscript, where the NOS-bridge is very likely central to function. First, we discuss the human DNA repair enzyme OGG1, which excises oxidized guanine bases from ROS-damaged DNA. In this example, the lysine of the detected NOS bridge is the key covalent catalyst. In other examples, the NOS bridge is either located at the active site and apparently regulates substrate binding or at the protein-protein interface of the nuclear egress complex of herpesviruses. All of the examples discussed

have a resolution better than 2 Å. The complete analysis of the pdb and a discussion of the found motifs will be in a separate publication.

We added at p. 9/10:

Analysis of the protein structure data base

We next asked whether the Cys-Lys NOS link exists in other proteins. We searched the protein structure database (www.rcsb.org) for juxtaposed lysine and cysteine side chains with unexplained electron density between the cysteine S atom and the lysine N atom. One requires true atomic resolution as well as sufficient occupancy to unambiguously identify the NOS bridge. As this chemical entity was not known, it could have been easily disregarded when interpreting electron density maps. When searching the protein data base, we could identify a multitude of proteins from all domains of life, which are highly likely to possess one or more NOS bridges. We identified several chemical and structural motifs, the discussion of which would be beyond the scope of this initial discovery report and will be presented in a separate publication (manuscript in preparation). That notwithstanding, we are briefly discussing three representative examples, where the detected NOS bridges are likely to impact biological function of a protein. In the human protein 8-oxoguanine glycosylase, an important enzyme involved in DNA base excission repair [33] under oxidative stress conditions and related to several cancers [34], an NOS bridge is observed between residues Cys253 and Lys249 (Fig. 5). As residue Lys249 is the key catalytic residue, formation of the NOS bridge is governing the catalytic and with that the biological function of this enzyme, either regulatory or as part of the catalytic mechanism. In other examples, NOS bridges are found at the substrate binding locale of the active site of enzymes or at a critical position at the binding interface of proteins forming the nuclear egress complex of herpesviruses thus regulating substrate or protein binding (ED Fig. 8).

We added a new Figure 5 with a key example:

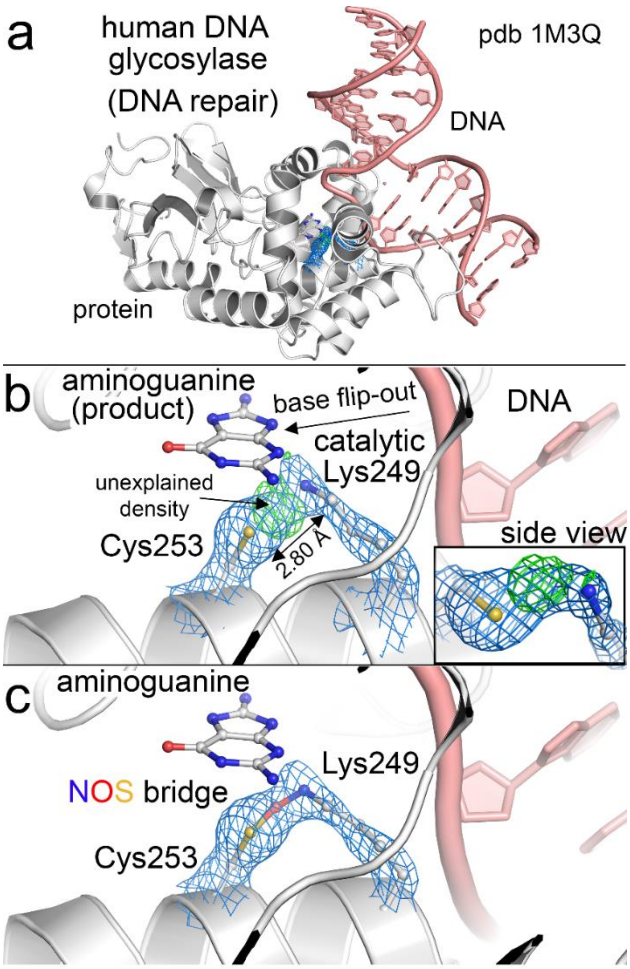
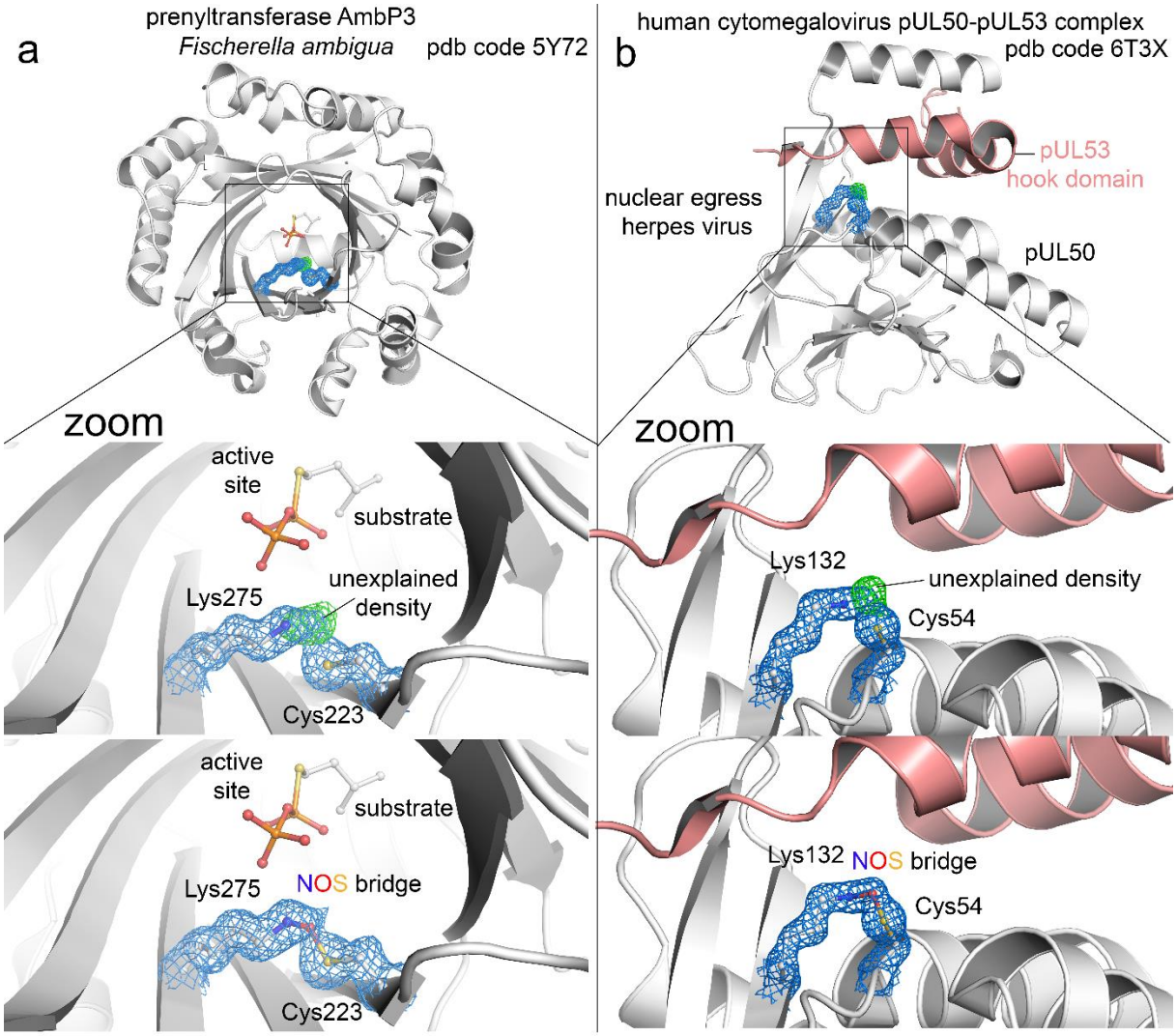


Figure 5. Structure of the human DNA repair enzyme 8-oxo-guanine glycosylase 1 (*h*OGG1) with a putative, hitherto undetected NOS bridge at the active site (pdb code 1m3q, 1.90 Å resolution). (**a**) Overall structure of *h*OGG1 in complex with DNA and product analog 8-amino-guanine. (**b**) Close-up view of the active site showing the product and residues Cys253 and Lys249. Residues Cys253 and Lys249 are superposed with the calculated 2mF-DFc electron density map (in blue, contour level 1.5σ). A strong, unexplained positive peak in the mFo-DFc difference electron density map (in green, contour level 3σ) is observed between the cysteine sulfur atom and the lysine nitrogen atom. The S-N interatomic distance (2.80 Å in the deposited structure) is too short for a H-bond interaction and too long for a direct S-N linkage. (**c**) Re-refinement of the deposited structure with a geometrically parametrized NOS bridge linking Cys253 and Lys249 resulted in a structural model with no remaining unexplained electron density (2mFo-DFc electron density map in blue, contour level 1.5σ; mFo-DFc difference electron density map in green/red, contour level ±3σ) suggesting the presence of an NOS bridge.

We further include a new ED Fig. 8 with two additonal examples.



Extended Data Figure 8. Representative examples of protein structures deposited in the protein data base likely containing an NOS bridge. For both examples (**a** & **b**), the overall structure is shown in cartoon representation in the upper panel highlighting the Lys-Cys linkage and providing the pdb code [41,42]. In the corresponding middle panels, the structure of the lysine-cysteine pair as deposited in the data base is shown enlarged including the calculated 2mFo-DFc (in blue, contour level 1σ) and mFo-DFc difference (in green, contour level +3σ) electron density maps. Note the pronounced positive difference electron density in between the lysine nitrogen atoms and the cysteine sulfur atoms indicating the presence of a covalent bridge. In the bottom panels, the re-refined structural models including the covalent Lys-Cys NOS bridges are shown with the corresponding 2mF-DFc electron density maps. The mFo-DFc difference electron density maps are shown in green and are contoured at 3σ. The calculated occupancies of the NOS bridges

amount to 62% (a) and 76% (b), respectively. Note the prominent location of the NOS bridge at either the substrate binding site (a) or the protein-protein binding interface (b).

Minor:

Please draw out the location of Lys8 in fig 2a.

Authors: The figure was revised as suggested.

Reviewer 2

A novel chemistry for protein crosslinking that has functional significance is a fundamental discovery, so this reviewer was excited to read and review the results. Overall the data look convincing, but many improvements to the manuscript can be made. Because the discovery is really quite remarkable, the paper deserves additional effort to perfect the presentation.

Authors: We would like to thank this reviewer for her positive assessment of our work and in particular for the many valuable improvements/corrections in science, language and style. We revised our manuscript accordingly.

p. 2 abstract: “protein crystallography of the protein”-- remove the first “protein” OK
p. 2: it doesn’t quite make sense to say that the crosslink was neglected “due to limited resolution in protein structure determination.” The authors identified the linkage in other structures, indicating that these structures were determined to sufficient resolution. The problem, at least in some cases, was that people passed over the evidence because they weren’t looking. OK statement omitted
p. 2 introduction: should be “three-dimensional structures” (plural) since each protein has its own structure. Also, what does “defined” mean here? Better would be “are formed and maintained.” OK
p. 2-3: can shorten to: “In addition to peptide bonds, only a few additional covalent bonds between amino acids contribute to…” This phrasing is also more accurate, since of course many other covalent bonds (the backbone N-C, C-C, and all covalent bonds in the side chain) also contribute to protein structure. But these others are not BETWEEN amino acids. OK
p. 3: The last sentence of the introdution is not completely clear. The second-to-last sentence described the reversibility of disulfides, but the last sentence seems to include the possibility of a larger variety of cysteine modifications and not just disulfides. To avoid confusion, the authors could perhaps say: “In addition to disulfides, a variety of other oxidized cysteine species serve as molecular sensors…” OK
p. 3 results: No essential information would be lost by shortening to: “Transaldolase (TAL) is a key enzyme in the pentose phosphate pathway. The Neisseria gonorrhoeae TAL ortholog (NgTAL) is a potential drug target for fighting gonorrhea, the sexually transmitted disease caused by this pathogen. When produced recombinantly, NgTAL showed almost no enzymatic activity. We hypothesized that the enzyme could be subject to activation by a redox event involving one or more of its three cysteines: Cys38, Cys87, and Cys90.” OK (changed in part)
p. 3-4: “supporting our initial hypothesis of a redox activation” should be removed, because the sentence is too long and the “In fact” at the beginning already indicates that the hypothesis was correct. OK
p. 4: There is no reason to lead the reader astray by mentioning “a putative disulfide-based redox switch.” The authors could instead write: “… is not altered by reduction, but differences were seen between the oxidized and reduced states in thermal unfolding.” However, see the next comment.

p. 14 Figure 2: It is not clear how the authors measure cooperativity in the thermal unfolding in panel D. If this is a quantitative measure, the method for calculating it should be given. To this reviewer, the slopes at the midpoint of the transitions look similar. The shapes of the folded baselines do look a bit different, but what does that mean exactly? This reviewer assumes that the differences in the folded and unfolded baseline signals between oxidized and reduced states in the melt are due to a protein concentration issue in this experiment, since panel C shows identical signals at 222 nm. At what temperature was the data in panel C collected? Why are the data in panel D not presented as molar (mean residue) ellipticity as in panel C? In general, the analysis (or at least the explanation) of the CD data should be improved. OK, section rewritten
p. 4: After correcting and condensing the description of the differences in the thermal melts, the authors should start a new paragraph without mentioning disulfides: “To determine which of the cysteines in NgTAL is involved in redox regulation, we constructed variants lacking each of the three cysteines. We predicted that substitution…” Disulfides are irrelevant to the story at this point. OK
p. 4: “We thus hypothesized that Cys38 might form an intermolecular disulfide bridge…” Here it is perfectly OK to mention disulfides, because now the hypothesis is explicit and not confusing/misleading. OK
p. 4: delete “seemingly viable” and change “had been” to “was” or “has been.”
p. 4: delete “using a set of different protein concentrations.” This information can be provided in the methods. OK
p. 4: Should be “The enzyme was predominantly a monomer under all conditions tested, falsifying our hypothesis of a redox switch involving a change in quaternary structure.” (The authors should be consistent about using the past tense to describe results of experiments, and it is sufficient to mention the minor dimeric population in the figure legend as is already done.) OK
p. 5: There is absolutely no need for riddles, conundrums, and enigmas! Or for misquoting Churchill. This section should read: “To further investigate the basis for redox activation, we crystallized NgTAL under oxidizing and reducing conditions. We determined structures of both states at true atomic resolution…” OK
p. 5: A quick search on Chemspider reveals that an N-O-S substructure is present in many molecules. But indeed they do seem to have higher oxidation states for the sulfur (but not always the same one as hydroxylamine-O-sulfonic acid). It also seems not quite right to compare the number of oxygens bound to the sulfur in HOSA with the number in the NgTAL linkage, since in cysteine the sulfur is bonded to a carbon, which is absent in HOSA. There are examples of the N-O-S substructure that are actually N-O-S-C, and in these cases the sulfur is typically bonded to either 2 or 3 oxygens. OK
p. 5: “(no other oxidants were present in solution)” can be omitted. OK
p. 6: It is not quite clear which side chains are close enough to constitute the hydrophobic patch in which the O2 is found. Ile14 is labeled behind the O2 and may be close enough to be relevant, but Leu4 and Val37 look too far away. Can the authors specify, perhaps in the figure legend, which residues make up the patch? Information added.
p. 6: There is no reason one should be confident about a suggestion, only about a conclusion. Perhaps rephrase: “… it is difficult at this point to determine the chemical mechanism for formation of the N-O-S linkage, especially as there is no precedent…” OK
p. 6: The authors should be consistent and use either N-O-S or NOS, but not both OK
p. 6: should be a comma after “do react with amines” OK
p. 6: rephrase: “Electronic structure calculations were carried out to compare…” (“in order to” means the same as just “to”) OK
p. 6: omit “safely” in “we can safely rule out” OK
p. 7: The paragraph on the electronic structure calculations seems to end without any clear suggestion for the mechanism. In contrast, the legend of extended Figure 3 does seem to make some suggestions. Even if the authors are not 100% sure, they can still improve the clarity of this paragraph by explaining why they disfavor various mechanisms outlined in the previous paragraph and which remain(s) as possible viable mechanism(s). Some of this information is present in the paragraph already, but not as coherently as could be. OK

p. 7: shorten: “To understand the structural basis for the decrease in NgTAL activity upon formation of the N-O-S linkage, we superposed the oxidized and reduced structures (Figure 4).” OK
p. 7: should be “strand-like structure” or “extended, strand-like structure” rather than “sheet-like structure.” OK
p. 7: should be “It is known for this enzyme and for many other enzyme classes… critical for catalysis, with small…” OK
p. 7: This reviewer found the comparison to GTPases distracting rather than helpful. The authors may want to consider deleting this comparison and ending the paragraph with the previous sentence. OK, we shortened this paragraph but kept the refernce to GTPases as the prime example for the loaded-spring mechanism
p. 7: The sentence “We next asked…” is problematic, since formation of the NOS linkage causes DE-activation of the enzyme, not activation.
p. 7-8: Simplify to “We thus generated Lys8Ala and Cys38Ser variants, which cannot form the crosslink. Lys8Ala showed a ~7 °C difference in melting temperature between the oxidized and reduced states, whereas Cys38Ser showed almost identical melting temperatures in the two states. This observation suggests that…” OK, rewritten.
p. 8: Perhaps start with: “The chemical identity of the N-O-S link appears well established on the basis of X-ray crystallography, but we tested whether this linkage could also be detected by mass spectrometry (MS). MS is routinely used for detection of disulfide bonds (28,29), and detection of N-O-S bonds would obviate the need for ultra-high resolution X-ray crystallography.” OK
p. 8: A lack of evidence is not evidence, so perhaps rephrase: “We were, however, unable to detect the crosslink between the two peptides bearing Lys8 and Cys38, and instead Cys38 was found predominantly in the sulfinic acid state (Extended Data Figure 5). It thus appears that the covalent NOS linkage is hydrolyzed under the acidic conditions used for sample preparation. The MS data are nevertheless informative, as they provide further support that the bridging atom is indeed oxygen. No modifications of Lys8 could be detected either in the oxidized or reduced enzyme, and Cys38 was detected as the unmodified thiol under reducing conditions.” OK
p. 8: shorten and modify: “The chemical instability of the NOS crosslink during sample processing, coupled with the absence of this species in the modifications searched during MS data analysis, explains why it has been missed in proteomics analyses so far.” The authors may be able to improve upon the suggested phrasing, but the point is that even if the crosslink had been stable, people still would have missed it in proteomics MS experiments unless they had known to specifically search for it. OK
p. 9: Should be: “We next asked whether the Cys-Lys NOS link exists in other proteins. We searched the protein structure database (www.rcsb.org) for juxtaposed lysine and cysteine side chains with unexplained…” OK
p. 9: Remove “Obviously” in “Obviously, one requires…” OK
p. 9: “we could identify numerous proteins from all domains of life”-- six proteins are shown in the supplementary figure. Were more than these identified? If not, perhaps delete “numerous,” which suggests at least tens if not hundreds (considering the size of the PDB). OK, rewritten
p. 9: change “most” to “many” in “most proteins are purified…” unless the authors can provide numbers regarding how many PDB entries were produced from crystals grown in the presence of reducing agents. Many secreted, cell surface, or organelle-localized proteins are disulfide bonded, and crystallographers working on those would not add reductant during crystallization.
p. 9: It is strange to read, “In conclusion…” and then right afterwards see a section headed “Conclusions.” OK; section removed
p. 9: Grammatically, one showcases something. One does not showcase that something. Also, the authors did not demonstrate in the other systems that the linkage has a role in either function or stability. Simplify to: “Our analysis shows that the NOS link is not unique to NgTAL and may contribute to function and stability in a diverse set of proteins.” OK
p. 9, Conclusions: “eliciting” is used incorrectly here. Increasing is the correct word. OK
p. 10: Again, it is true that lability causes a problem for MS, but it is also true that people need to know

what to look for to find modifications by MS (and other techniques). That is why this paper is so important-- it reveals a completely new and unexpected thing to look for. OK
p. 13, Figure 1A: What is the meaning of the double arrows going from thiol and sulfenic acid (third row in panel a) to intermolecular disulfide (second row in panel a)? Yes, thiols can be oxidized to disulfides, but this is a redox reaction, and unlike the other processes shown in the third row, no other redox species are shown to be involved. Also, these arrows are confusing because they don't show where the red-sphere species comes from. We wanted to connect oxidation with disulfide formation
p. 16, Figure 3: The distances are in very small type in panels c and d. There is a general problem in all the figures of the paper that the font sizes vary a lot, and not always does the size reflect the importance of the information. OK, fonts identical but figures could have been resized

Extended data:
p. 4: The yellow on a gray background is very difficult to see. This reviewer asked some much younger people (with better eyesight), and they agreed. OK, background removed
p. 7: The size of the text is too small in the table, and the chromatography peaks and labels are miniscule.

OK, text was resized.

Reviewer 3

1) Overall, the structure of NgTAL is beautifully resolved at subatomic resolution and shows unique features compared to the previously determined NgTAL structure (PDB 3CLM, 1.14 Å). The proposed assignment of the linkage atom seems reasonable due to the subatomic resolution. However, whether this species is biologically relevant or an artifact remains questionable. The reasons for this are described below.

First, no additional evidence (besides the crystal structure) appears to support the existence of the NOS linkage, raising the question of whether the observation is due to radiation damage. To rule this out, the authors need a solid MS analysis, at a minimum, to compare the protein crystal samples before and after X-ray data collection. It seems that the lack of an NOS linkage in the LC-MS analysis was rationalized due to the acidic condition when running LC-MS. Many other current MS techniques could overcome this issue or simply a change of buffer/column. If this NOS linkage exists in the crystal structures that authors use to generalize the wide distribution of NOS linkage across all kingdom, some of the ascribed enzymes were likely resolved under acidic conditions, such as PDB 5Y72 and PDB 5YZP, and, as such, would not be expected to retail the proposed NOS crosslink.

Authors: We have indeed tested different conditions (basic pH) and columns (and add this info briefly in the MS section) but could not unambiguously identify the crosslink. It appears that the native protein conformation stabilizes the NOS bridge (at least in the protein we studied). We have started with native MS and MS of undigested protein samples and have (still preliminary) promising results. We believe that the development of robust MS protocols is important but would like to communicate this separately.

We can definitely exclude that the NOS bridge is due to "radiation damage". We have now measured crystals of the oxidized enzyme at our local in-house rotating anode (Rigaku Micromax-003), which deposits a very small dose and still do observe the NOS bridge.

We state at p. 4/5:

This crosslink was reproducibly observed for numerous proteins crystals obtained under different non-reducing crystallization conditions and also for a low-dose dataset collected at an in-house rotating anode excluding the possibility that formation of the crosslink results from “radiation damage” during data collection at the high-energy synchrotron beamline (ED Fig. 1a-d).

The in-house dataset was deposited at the pdb and the results are shown in Extended Data Fig. 1d.

2) Second, the activity observed in the kinetic assay containing DTT is not enough to support biological relevance for the NOS structure. As the authors mention in the MS analysis, modifications have been observed on the cysteines, suggesting that the purified enzyme in the absence of reducing reagent has already undergone oxidative damage during sample preparation. It is not surprising that low catalytic activity is observed for this protein sample. Additionally, since the kinetic assay is a coupled assay using triosephosphate isomerase (TIM) and sn-glycerol-3-phosphate:NAD+ 2-oxidoreductase (G3PDH), a control experiment indicating whether DTT impacts the coupling reagents is lacking in the manuscript.

Authors: We do not observe any other chemical modification in the numerous X-ray crystallographic structures of the oxidized enzyme other than the NOS bridge. The two other cysteines are always in the unmodified thiol form. Hence, it seems justified to correlate enzymatic activity with the presence or absence of the NOS bridge.

The auxiliary enzymes are not impacted by the presence of DTT. We use this assay in several projects and have done all relevant controls. Also, the kinetic data on the redox-insensitive variant Cys38Ser revealed virtually identical kinetic constants for the oxidized and reduced (1 mM DTT) enzyme. This would be impossible, if DTT would impact the activity of the auxiliary enzymes. We have added this info to the methods section.

Online methods section: We established that the activity of the auxiliary enzymes is not impacted by the addition of DTT.

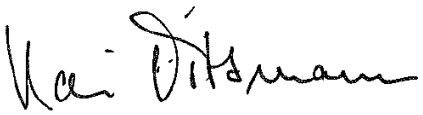
3) A minor point, the crosslink between cysteine and other residues, such as Trp, Tyr, Arg and Glu, is not unique to galactose oxidase. These cysteine crosslinks also exist in enzymes such as cysteine dioxygenase (McCoy, J. G., et al. 2006 Proc Natl Acad Sci, 103, 3084-3089.), lysine ε-oxidase (Okazaki, S. et al. 2013 J Biochem, 154, 233-236.) and quinohemoprotein amine dehydrogenases (Datta, S., et al. 2001, Proc Natl Acad Sci 98, 14268-14273). It is even more diverse if small proteins and peptides are included, such as sactipeptides (Balty, C. et al. 2019 J Biol Chem, 294, 14512-14525, & Kawulka, K. E., et al. 2004 Biochemistry, 43, 3385-3395.) and thiopeptides (Kelly, W. L. et al. 2009 J Am Chem Soc 131, 4327-4334.). The statement in the abstract that “native crosslinks with neighboring amino acids other than cysteines have not been demonstrated for proteins with one notable exception” is not accurate.

Authors: We agree and have rephrased this sentence.

Abstract: Although oxidized cysteine species are highly reactive and may form covalent conjugates with tyrosines in the active sites of some redox enzymes [7,8], regulatory switches with covalent crosslinks other than disulfides have not been demonstrated.

With the additional results presented here and taking into account that we are able to address all other issues raised by the reviewers, we hope you will find our manuscript now suitable for eventual publication in *Nature*.

Most sincerely, on behalf of all coauthors,



Kai Tittmann

Reviewer Reports on the First Revision:

Ref #1

I thank the authors for their efforts to address the concerns raised and also the very clear markup of the changes.

Regarding the first issue of reversibility of the modification:
The statement that "the reduced enzyme converts into the oxidized form over a couple of days as confirmed by X-ray crystallography (see below)" is important, especially as it also, depending on the sample conditions provides information on the oxidant (and would in that way also reduce the second concern below).

however, I could find no further data, description or figures for this. How many days, what was the sample condition (cell lysate, purified protein, buffer conditions etc.). Also, after that statement I expected to see some experimental data supporting that claim and a figure of the reoxidized structure.
Importantly, as far as I could find, the authors claim reoxidation but do not mention the other key point of the question, is the reoxidized protein re-inactivated. This is an obvious measurement but I could not find it described in the study.

Regarding the concern of in vivo relevance:
The bioinformatic study of conservation of the crosslinked residues in transaldolases is very much appreciated. Two clusters of sequences were found to contain these residues, this is nice, however one must also have some discussion as to how conserved the sequences in these clusters are overall to be able to judge whether the conservation of these two residues is significant (i.e. under some form of evolutionary pressure).
The work on NmTAL is very much appreciated, however, it also raises some additional concerns. While the direction of the effect is similar, the magnitude is very different. NmTAL shows a modest 2.3 -fold increase in kcat and 1.5 fold decrease in Km upon reduction giving a kcat/Km increase of only 3.46 (which the authors have rounded to "4-fold" in table ED1). This is to be compared to 30-fold, 2-fold and 60-fold in NgTAL, respectively.
While different enzymes are commonly observed to have different catalytic parameters the influence of activity by reduction is very modest for NmTAL and more than an order of magnitude smaller compared to NgTAL, which appears puzzling if the same regulatory mechanism is proposed. This concern is aggravated considering that NmTAL and NgTAL are 93% identical on the protein sequence level, something which would normally suggest very similar catalytic properties. Looking at the catalytic parameters (ED table 1). The main difference between NmTAL and NgTAL is the very low activity in oxidized NgTAL, which is not the case for oxidized NmTAL.
In some way the possibility that the original oxidized NgTAL sample has undergone some form of adventitious oxidative damage during sample preparation should be excluded. As an absolute minimum this must include activity measurements of the protein both after reduction and after reoxidation (see above comment).

Regarding the mechanistic discussion, if O2 is indeed shown to be the oxidant, (see above), this is now much more relevant. To figure this out should now be trivial given that the authors can reoxidize the sample by simply waiting. Performing the same experiment in an anaerobic environment would provide the key data on the oxidant.

Regarding the PDB-database mining. I agree it is relevant to bring up these examples. However, without any biochemical data the wording is too strong to the point of being misleading and must be significantly toned down, for example: "an NOS bridge is observed between residues Cys253 and Lys249 (Fig. 5). As residue Lys249 is the key catalytic residue, formation of the NOS bridge is governing the catalytic and with that the biological function of this enzyme" -A NOS bridge has not been established, there is no data if it influences activity and there is no data to show that the crosslink is not an artefact.

Ref #2

The authors went to much effort to address the reviewers' comments, and the manuscript is improved. In the opinion of this referee, the functional impact of the N-O-S cross-link in various biological systems can be investigated in the future outside the context of this manuscript. The strength of this work is the protein chemistry and the potentially broad implications.

One small point-- in the added extended data Figure 6, what is meant by "motif 1" and "motif 2"?

It is the same motif (homologous, with all key amino acids conserved). Two clusters of organisms share this one motif.

Ref #3

In the revised manuscript the authors have addressed one important question, regarding the possibility of radiation damage during x-ray measurements.
While they did not pursue the suggested use of mass spectrometry for structure validation, they did employ an alternative, low-dose, in-house beam that supports the reported NOS bridged structure.

The authors have also shown that the impact of DTT on kinetic properties is not due to inhibition of coupling enzymes employed in kinetic assays.

Their bioinformatic analysis as well as the re-analysis of electron density maps of previously published structures is also quite informative, and supports a new type of NOS modification that protein biochemists and structural biologists have overlooked.

The biological relevance of the NOS cross-link is still not clearly demonstrated, though this is implied from the title and stated in the conclusions.

Despite some unresolved issues regarding biological function, the overall findings represent a beautiful crystallographic discovery.

Author Rebuttals to First Revision:

Referee 1

1) Regarding the first issue of reversibility of the modification:

The statement that “the reduced enzyme converts into the oxidized form over a couple of days as confirmed by X-ray crystallography (see below)” is important, especially as it also, depending on the sample conditions provides information on the oxidant (and would in that way also reduce the second concern below).

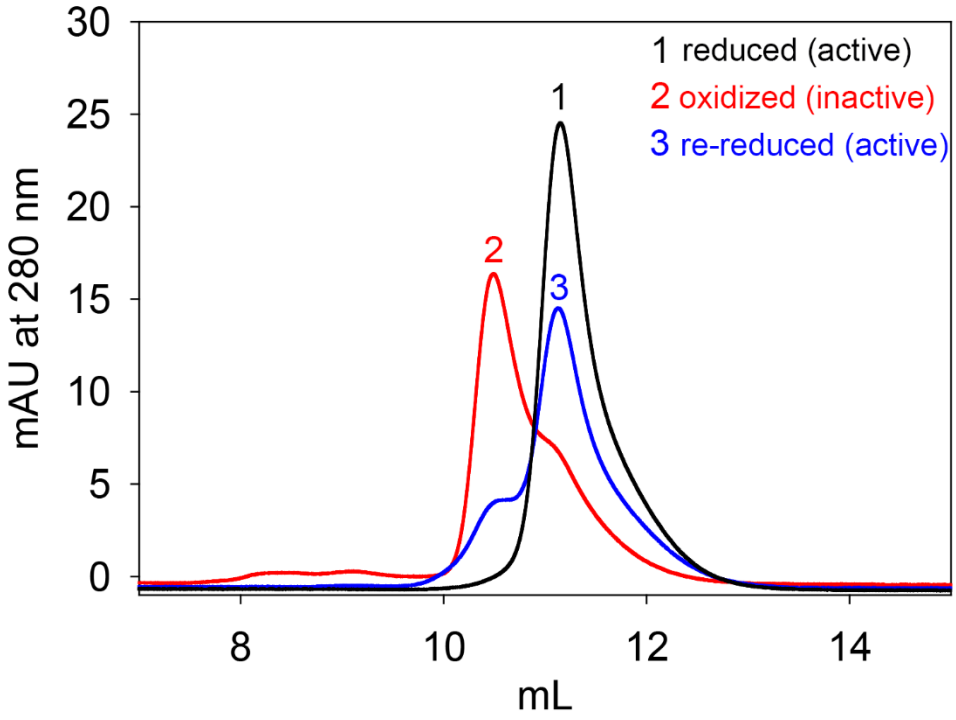
However, I could find no further data, description or figures for this. How many days, what was the sample condition (cell lysate, purified protein, buffer conditions etc.). Also, after that statement I expected to see some experimental data supporting that claim and a figure of the reoxidized structure.

Importantly, as far as I could find, the authors claim reoxidation but do not mention the other key point of the question, is the reoxidized protein re-inactivated. This is an obvious measurement but I could not find it described in the study.

Authors: We have been adding the requested data and demonstrate that the redox switch is indeed reversible.

We now state at page 3:

This redox activation is essentially reversible, the reduced enzyme converts into the inactive, oxidized form over a couple of days that can be re-activated by reduction (SI Fig. 1).



SI Figure 1. Size-exclusion chromatographic analysis of *Ng*TAL (1 mg/ml in all samples) in the oxidized (enzymatically inactive) and reduced (enzymatically active) state using an analytical SEC S75 column. Freshly prepared and enzymatically active NgTAL in the reduced state (sample 1, black) elutes at ~11.1 min. The reduced enzyme was separated from excess reductant DTT using a desalting G25 column and allowed to oxidize at 6 °C. With progressing oxidation over a couple of weeks, the enzyme became inactive (v/E = 0.22 ± 0.01 s^{-1}). The oxidized form (sample 2, red) elutes at ~10.4 min with some minor fraction of the reduced form present. Re-reduction of the oxidized enzyme by addition of 20 mM DTT led to a reactivation of the enzymatic activity (v/E = 25.0 ± 2.2 s^{-1}). The thus obtained re-reduced enzyme (sample 3, blue) elutes at ~11.1 min similar to the freshly prepared reduced enzyme.

A likewise separation of the oxidized and reduced enzyme was obtained under preparative conditions using a preparative SEC S75 column.

2) Two clusters of sequences were found to contain these residues, this is nice, however one must also have some discussion as to how conserved the sequences in these clusters are overall to be able to judge whether the conservation of these two residues is significant (i.e. under some form of evolutionary pressure).

Authors: We believe that such a discussion would be too speculative as one cannot exclude that hitherto undetected lysine-cysteine redox switches at other positions in the transaldolase sequence space might

exist. We agree that this is an important aspect but feel that this analysis goes beyond the scope of the current study.

3) The work on *Nm*TAL is very much appreciated, however, it also raises some additional concerns. While the direction of the effect is similar, the magnitude is very different. NmTAL shows a modest 2.3 -fold increase in kcat and 1.5 fold decrease in Km upon reduction giving a kcat/Km increase of only 3.46 (which the authors have rounded to “4-fold” in table ED1). This is to be compared to 30-fold, 2-fold and 60-fold in NgTAL, respectively.

While different enzymes are commonly observed to have different catalytic parameters the influence of activity by reduction is very modest for *Nm*TAL and more than an order of magnitude smaller compared to *Ng*TAL, which appears puzzling if the same regulatory mechanism is proposed. This concern is aggravated considering that NmTAL and NgTAL are 93% identical on the protein sequence level, something which would normally suggest very similar catalytic properties.

Authors: We agree that this finding is somewhat unexpected. On the other hand, the two proteins are of different origin and it is well established that even closely related orthologs may have different enzymatic properties. The kinetic differences of the oxidized enzymes translate into thermodynamic barriers that differ by ~1 kcal/mol, which is little considering that the allosteric redox switch controls the enzymatic activity at the active site through subtle repositioning of catalytic residues.

We added at page 8:

Despite the high degree of similarity of *Nm*TAL and *Ng*TAL on the sequence level, the enzymatic activity of the two enzymes in the oxidized state are different suggesting a subtle crosstalk between the allosteric and active site.

Also, the kinetic analysis of *Nm*TAL in different redox states is technically difficult as the oxidized and reduced forms cannot be quantitatively separated by chromatography methods as one can do in case of the title enzyme *Ng*TAL (see SI Fig. 1). Thus, we cannot exclude that the sample with the oxidized enzyme contains traces of the highly active reduced form. We refer to this circumstance at various places in the manuscript

We state in the legend to ED Table 1:

The catalytic constant of *Nm*TAL in the oxidized form represents an upper limit as oxidized and reduced species cannot be quantitatively separated by chromatographic methods as in case of *Ng*TAL.

4) Regarding the mechanistic discussion, if O2 is indeed shown to be the oxidant, (see above), this is now much more relevant. To figure this out should now be trivial given that the authors can reoxidize the sample by simply waiting. Performing the same experiment in an anaerobic environment would provide the key data on the oxidant.

Authors: Oxygen was the only potential oxidant in solution so we think it is reasonable to suggest that it is critical for NOS bridge formation. In strong support of this claim, we could even trace dioxygen in the high-resolution crystallographic structure. As we have stated in the previous letter, we have started to systematically analyse the reactivity of the redox switch with different reductants and oxidants but would like to address this point in a separate publication as this goes beyond the scope of the current manuscript.

5) Regarding the PDB-database mining. I agree it is relevant to bring up these examples. However, without any biochemical data the wording is too strong to the point of being misleading and must be significantly toned down, for example: “an NOS bridge is observed between residues Cys253 and Lys249 (Fig. 5). As residue Lys249 is the key catalytic residue, formation of the NOS bridge is governing the catalytic and with that the biological function of this enzyme” -A NOS bridge has not been established, there is no data if it influences activity and there is no data to show that the crosslink is not an artefact.

Authors: We have rewritten this section and state now at page 9:

As residue Lys249 is the key catalytic residue that forms a Schiff-base linkage with the DNA, formation of the NOS bridge - if relevant in vivo – could potentially regulate the enzymatic activity through e.g. inactivation of the essential lysine. In other examples, NOS bridges are found at the substrate binding locale of the active site of enzymes or at a critical position at the binding interface of proteins forming the nuclear egress complex of herpesviruses thus potentially regulating substrate or protein binding (ED Fig. 8).

We would nonetheless emphasize that the relevant biochemical data in support of a redox switch in Ogg1 are existing but will be discussed elesewhere.

Editorial requests

We have reorganized the ms to meet the demands in terms of space allocation, allowed characters for different sections etc.

1) The title was shorted to

“A lysine-cysteine redox switch with a covalent NOS bridge regulates enzyme function”

2) We moved the mass spec section into the SI file as supplementary results as well as the related figure.

3) We shortened the text at different places and thus reduced the word count to ~3000.

4) We moved Figure 1 to the Extended Data to save space (now 4 figures instead of 5).

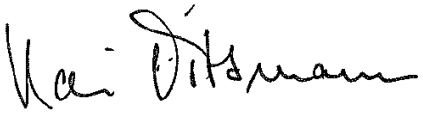
5) We created a Supplementary Information file containing Supplementary Results (mass spec) and three supplementary figures.

6) The Extended Data now contain 8 figures and 2 Tables, each in one side format.

7) Information about statistics is provided in the legends to the relevant figures and tables.

With the additional results presented here and revisions made, we hope you will find our manuscript now suitable for eventual publication in *Nature*.

Most sincerely, on behalf of all coauthors,



Kai Tittmann

Reviewer Reports on the Second Revision:

Ref #1

I again thank the authors for their efforts to address the concerns raised and also the very clear markup of the changes.

The authors have addressed (almost) all of my comments and concerns and put in a lot of effort doing so. Particularly the reversibility and the now very likely involvement of oxygen puts the study on more solid ground.

These are indeed interesting observations and i support publication.

Ref #2

N/A

Ref #3

N/A