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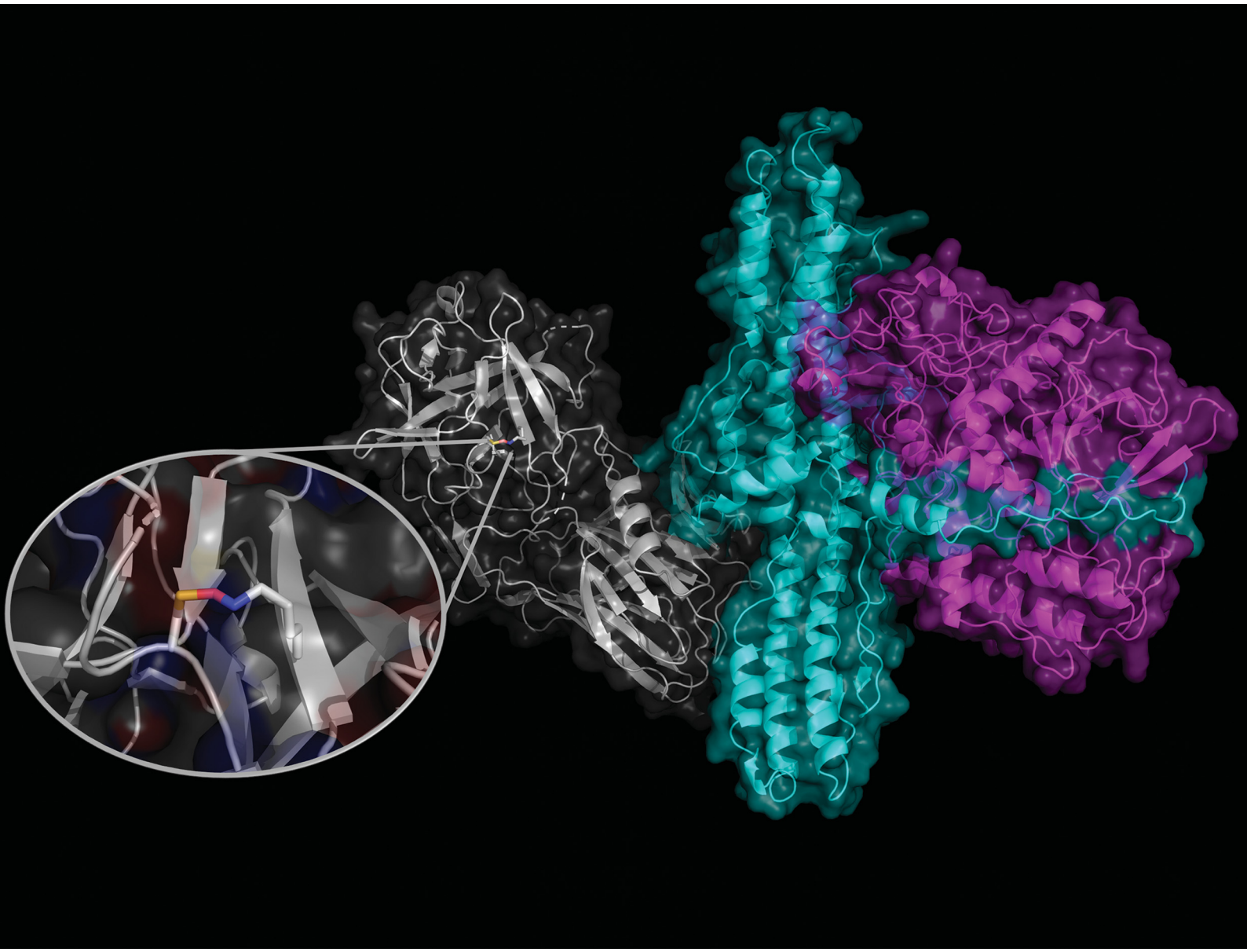

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Functional implications of unusual NOS and SONOS covalent linkages found in proteins

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The tertiary and quaternary structures of many proteins are stabilized by strong covalent forces, of which disulfide bonds are the most well known. A new type of intramolecular and intermolecular covalent bond has been recently reported, consisting of the Lys and Cys side-chains linked by an oxygen atom (NOS). These post-translational modifications are widely distributed amongst proteins, and are formed under oxidative conditions. Similar linkages are observed during antibiotic biosynthesis, where hydroxylamine intermediates are tethered to the sulfur of enzyme active site Cys residues. These linkages open the way to understanding protein structure and function, give new insights into enzyme catalysis and natural product biosynthesis, and offer new strategies for drug design.

Introduction

Protein tertiary and quaternary structures are often stabilized by covalent bonds which cross-link amino acid side-chains which are non-adjacent in the primary sequence but close together in the folded structure. In peptides, these cross-links can be used to stabilize a desired conformation. Such peptides are typically referred to as ''stapled''.[1]

By far the best known of these cross-links are disulfide bonds between two cysteine residues, which form under oxidizing conditions.[2] Disulfide bonds are prevalent in extracellular proteins, as these encounter more oxidizing conditions than in the cell. Disulfide bonds have also been implicated in protein folding.[3] Disulfide bonds can be readily reduced to cysteine residues in the sulfhydryl form. Isopeptide bonds have also been described, where the Lys and Asn amino acid residue side-chains[4,5] are covalently joined together by a peptide (amide) bond or where Lys side-chains are covalently linked with the side-chains of Glu residues[5] or the C-terminus.[6,7] Important examples of this process are ubiquitination[6,7] and cross-linking of proteins upon exposure to organophosphorus nerve agents and pesticides.[5]

In 2016 a new type of protein covalent bond was described by Ruszkowski and Dauter,[8] in which a cysteine sidechain was

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Matthew D. Lloyd read Biological Chemistry at the University of Leicester, followed by a DPhil (PhD) at the University of Oxford, working on antibiotic biosynthesis. After several years of working as a post-doctoral researcher at Brown University, USA and Oxford, Matthew joined the University of Bath in 2002. He is currently Senior Lecturer (Associate Professor) in the Department of Life Sciences at Bath. His research focusses on enzymes, enzyme kinetics, and development of enzyme inhibitors using techniques from Medicinal Chemistry, Chemical Biology, and Biochemistry. He is also International Instructor, 5th Degree Black Belt in Ch'ang-Hon (ITF) Taekwon-do.

Kyle S. Gregory

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covalently linked to that of a lysine residue *via* a short spacer. This was identified using X-ray crystallography in a histidinol phosphate phosphatase structure at 1.32 Å resolution. This new covalent bond stabilized dimer assembly, and the short spacer was proposed to be a methylene group based on the high resolution crystal structure and mass spectrometric analysis.[8] At the time it was speculated that this linker arose by reaction of carbon dioxide with the Lys side-chain followed by cross-linking and reduction,[8] but this is extremely unlikely. Furthermore, an examination of the Protein Data Bank (PDB) in 2015[9] showed that there were 14 examples where a methylene bridge could be modelled into positive electron density between a Cys and Lys residue. Many subsequent reports have also observed clear electron density between Lys and Cys sidechains in different high resolution crystal structures, and they propose an oxygen atom as the linker (Table 1).[2,10–14] These covalent linkers have also been reported in *Clostridium botulinum* neurotoxin structures (Fig. 1) (specifically in the cell binding domain) determined by the Acharya laboratory.[12–15] Differentiating between a methylene and an oxygen bridge in proteins is non-trivial since these cannot be easily distinguished by mass spectrometry, given the unknown protonation states, or by electron density maps obtained by X-ray crystallography.[10,16,17] The identity of the bridge can only be inferred indirectly[18] unless very high-resolution crystallographic electron density maps (ideally beyond 1.0 Å resolution) are available[17,19] or by sulfur K-edge X-ray absorption spectroscopy data.[20]

Methylene bridged species

Methanal (Scheme 1, **1**) is a ubiquitous environmental pollutant and is produced as a metabolic intermediate in normal cells and cells undergoing oxidative stress.[21] The earliest observation of methylene bridge **5** formation by X-ray crystallography was in histidinol phosphate phosphatase crystals treated with methanal **1**.[8] They proposed formation by the well-known Mannich reaction[9] (Scheme 1) in which methanal **1** reacts with a deprotonated Lys **2** side-chain to generate an iminium ion **4**, which readily undergoes nucleophilic attack by the deprotonated Cys **3** sulfur. The formation of methylene bridges **5** in other enzymes has also been proposed.[15,16] However, it has been argued that histidinol phosphate phosphatase is a special case because the crystals are treated with methanal **1**;[8,16,18] this is not the case for the majority of proteins where these bridges are observed.

Several studies using model peptides have been carried out.[1,22] Metz *et al.*, demonstrated that methanal **1** reacts with the Lys **2** amino side-chain to give the corresponding hydroxymethyl derivative, although some Lys residues were remarkably difficult to derivatize for reasons that are unclear. Methanal **1** also derivatized the N-terminal amino group of peptides as well as the sidechains of Cys **3**, Arg, His or Trp residues to give hydroxymethyl derivatives (Cys **3** residues) or in some cases the corresponding imine (Lys **2** and Trp residues).[22] No intramolecular methylene bridges within these peptides were observed upon methanal **1** treatment. Pre-treatment of glycine with methanal **1** led to rapid formation the iminium adduct. This adduct reacted with the side-chains of several different residues within peptides to form methylene-bridged adducts, but crucially Cys **3** residues were unreactive. Li *et al.*,[1] noted that peptides containing Lys **2** and Tyr or Arg were generally quite easy to cross-link using methanal **1**, whilst cross-linking of Lys **2** and Cys **3** residues was relatively difficult to achieve. They also noted that methylene bridges between Cys **3** and Lys **2** were labile and could be easily hydrolysed.[1]

Formation of methylene bridges has also been described between N^{α}-Boc-amino acids and short peptides containing Lys **2**, Trp, Cys **3**, and His and deoxynucleosides and deoxynucleotides in the presence of methanal **1**, although cross-linking between Lys **2** and DNA bases was reversible.[21] Deoxythymidine was unreactive, but methylene-bridged adducts to other DNA nucleotides were formed. Carcinogenicity of methanal **1** is suggested to arise by cross-linking of Lys-containing histones with DNA.

K. Ravi Acharya

Ravi Acharya received his PhD degree from Bangalore University in India and then studied structures of macromolecules as a postdoctoral researcher in the Laboratory of Molecular Biophysics at Oxford with Louise Johnson, David Phillips, and David Stuart. He established a structural biology group at University of Bath in 1990 and became a Professor of Structural Molecular Biology in 1998. The main focus of his research has been in understanding the structural basis of protein function in normal and disease processes. As part of this research, he has pursued structural studies on botulinum neurotoxins with a view to understand their function.

Oxygen-bridged species

There has been considerable debate about the identity of the bridging atom.[10,16,18,23] Wensien *et al.*, 2021[2] noted that formation of the NOS bridge took place under oxidative conditions, with oxygen as the putative oxidizing agent. Moreover, the NOS bridge could be reduced using DTT or TCEP.

Reanalysis[10] of the original report of this novel covalent bond[8] suggests the electron density observed in the crystal structure is more consistent with the presence of an oxygen than a $-CH_2-$ group, as does reinterpretation[10] of the mass spectrometric data. A subsequent high-resolution X-ray structure of *Neisseria gonorrhoeae* transaldolase[2] was also consistent with the presence of a bridging oxygen atom rather than a $-CH_2-$ group. Careful analysis also showed that the observed NOS bridge was unlikely to have resulted from radiation

Table 1 Proteins containing NOS and SONOS linkages. Table is adapted from Rabe von Pappenheim *et al.*, 2022[11]

Protein	NOS or SONOS	Organism	PDB codes
Arginine decarboxylase[28]	NOS	*Paramecium bursaria* chlorella virus 1	2nv9
DNA polymerase[29]	NOS	Bacillus phage phi29	2py5
Nuclear egress protein 2, nuclear egress protein 1[30]	NOS	Human cytomegalovirus (strain ad169)	6t3x
Fibre protein[31,32]	NOS	Human adenovirus type 19 and 37	1uxb, 2wgu
Fibre protein[33]	NOS	Human adenovirus type 26	6qu8
RNA-directed RNA polymerase[34]	NOS	Hepatitis C virus subtype 1a	3qgi
Main protease (M^{PRO}) a.k.a. 3C-like proteinase[27,35–39]	SONOS	Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	7zb6, 7zb7, 7zb8, 7uua, 7uub, 7uuc, 7uud, 7uue, 6xmk, 7d1m, 7jr4, 6y2f, 3snd
Aldehyde dehydrogenase	NOS	*Listeria monocytogenes*	3k9d
Carbohydrate binding family 6/xylanase[40]	NOS	*Clostridium thermocellum*	2y8k
Botulinum neurotoxin serotype A (subtypes A1, A2, A5, and A6)[13,14,41,42]	NOS	*Clostridium botulinum*	3fuo, 5mk6, 7z5t, 7z5s, 8alp, 6twp
3-Deoxy-D-arabinoheptulosonate-7-phosphate synthase[43,44]	NOS	*Mycobacterium tuberculosis*	5e5g, 3rzi
3-Deoxy-manno-octulosonate cytidylyltransferase	NOS	*Acinetobacter baumannii*	4fcu
Dihydrodipicolinate synthase	NOS	*Pseudomonas aeruginosa*	3na8
dTDP-4-dehydro-6-deoxyglucose 3-epimerase[45]	NOS	*Streptomyces bikiniensis*	4hn1
DUF4468 domain-containing protein	NOS	*Parabacteroides distasonis*	4jhy
Glutaryl-CoA dehydrogenase[46]	NOS	*Burkholderia pseudomallei*	3gqt
Putative hydantoin racemase[47]	NOS	*Klebsiella pneumoniae* subsp. Pneumoniae	3qvl
Interferon-activable protein 204[48]	NOS	*Fischerella ambigua* utex 1903	5y72
Isochorismatase family protein	NOS	*Desulfovibrio vulgaris*	3hu5
KDPG (2-keto-3-deoxy-6-phosphogluconate) aldolase (putative)[49]	NOS	*Oleispira antarctica*	3vcr
LPG2147 (MavC)[50]	NOS	*Legionella pneumophila*	6ulh
Listeriolysin regulatory protein[51]	NOS	*Listeria monocytogenes* egd-e	6eut
Maleylpyruvate hydrolase[52]	NOS	*Sphingobium* sp. (strain nbrc 103272/syk-6)	6jvv
Metal binding protein RUMGNA_00854	NOS	*Ruminococcus gnavus*	3u7z
Methyl transferase[53]	NOS	*Burkholderia glumae*	5je5
New Delhi metallo-β-lactamase 1 (NDM-1) mutant[24]	NOS	*Klebsiella pneumoniae*	7ct2
Nitroreductase family protein	NOS	*Clostridium novyi*	3g14
Oxaloacetate decarboxylase 2[54]	NOS	*Vibrio cholerae*	2nx9
3-Oxoacyl-[acyl-carrier-protein] synthase 2[55]	NOS	*Escherichia coli*	3ho9
Penicillin-binding protein 4[56]	NOS	*Staphylococcus aureus*	6dz8
Periplasmic divalent cation tolerance protein[57]	NOS	*Thermotoga maritima*	1vhf
Pyruvate-formate lyase-activating enzyme	NOS	*Bacteroides vulgatus* ATCC 8482	3can
Regulatory protein[58]	NOS	*Salmonella typhimurium* (strain 14028s/sgsc)	6ie9
Ribonucleotide reductase R2[59]	NOS	*Escherichia coli*	1mxr
L-Ribose isomerase[60]	NOS	*Acinetobacter*	4q0p, 4q0q, 4q0s
SH2 domain-containing protein[61]	NOS	*Legionella longbeachae* serogroup 1	6e8h
Sucrose hydrolase[62]	NOS	*Xanthomonas axonopodis* pv. Glycines	3cze, 3czg
Sucrose hydrolase[63]	NOS	*Xanthomonas campestris* pv. Campestris	2wpg
Sulfur transferase DsrE	NOS	*Allochromatium vinosum*	2hy5
Transaldolase[2]	NOS	*Neisseria gonorrhoeae*	6zx4, 6zwj, 6zwh, 7b0l
DD-Transpeptidase[64,65]	NOS	*Streptomyces* sp.	1es2, 1es5, 1esi, 1skf
UPF0254 protein MJ1251[66]	NOS	*Methanocaldococcus jannaschii*	3wva, 3wvb
S-Ureidoglycine aminohydrolase cupin domain-containing protein[67]	NOS	*Thermotoga maritima*	1o5u
Calexcitin[68]	NOS	*Doryteuthis pealeii*	4ndb
Calexcitin[69]	NOS	*Loligo pealeii*	2ccm
Farnesyl diphosphate synthase	NOS	*Trypanosoma cruzi*	6sdp
Fluorescent protein Dronpa[70]	NOS	*Echinophyllia* sp. Sc22	2iov
Histidinol-phosphate phosphatase (MtHPP)[8]	NOS (originally interpreted as a methylene bridge)	*Medicago truncatula*	5eqa
F-actin-capping protein[71]	NOS	*Gallus gallus*	3aa0, 3aa1, 3aa6, 3aa7
Focal adhesion kinase 1[72]	NOS	*Gallus gallus*	6cb0
Glycolipid transfer protein[73]	NOS	*Bos taurus*	1tfj
Homeobox protein Hox-A9[74]	NOS	*Mus musculus*	1puf
IfI204[75]	NOS	*Mus musculus domesticus*	5yzp

Table 1 (*continued*)

Protein	NOS or SONOS	Organism	PDB codes
Kelch-like ech-associated protein 1[76]	NOS	*Mus musculus*	6qmj
Profilin-2[77]	NOS	*Mus musculus*	2v8f
Tubby protein[78]	NOS	*Mus musculus*	1i7e
cAMP and cAMP-inhibited cGMP 3′,5′-cyclic phosphodiesterase[79]	NOS	*Rattus norvegicus*	3qpo
Galectin-1	SONOS	*Rattus norvegicus*	4ga9
Glutathione S-transferase A1-1 hybrid[80]	NOS	*Homo sapiens*/*Rattus norvegicus*	5ld0
Rabphilin-3A[81]	NOS	*Rattus norvegicus*	4np9
S-Adenosylmethionine synthase isoform type-2[82]	NOS	*Homo sapiens*	6faj, 6fbp, 6g6r
Barrier-to-autointegration factor[83]	NOS	*Homo sapiens*	6usi
Butyrophilin subfamily 3 member A1[84]	NOS	*Homo sapiens*	6ism
Casein kinase I isoform δ[85]	NOS	*Homo sapiens*	6f1w
Casein kinase I isoform γ-3	NOS	*Homo sapiens*	2izu
Cysteine desulfurase, mitochondrial	NOS and SONOS	*Homo sapiens*	6uxe, 6w1d, 6wi2, 6wih
Density-regulated protein[86]	NOS	*Homo sapiens*	6vpq
Diphosphoinositol polyphosphate phosphohydrolase 1[87]	NOS	*Homo sapiens*	6pck, 6pcl
Dual specificity protein kinase CLK1[88,89]	NOS	*Homo sapiens*	6i5h, 6i5i, 6ft9
Early activation antigen CD69[90]	NOS	*Homo sapiens*	1e8i
Gem-associated protein 5[91]	NOS	*Homo sapiens*	5gxh, 5gxi, 5tha
Glutathione S-transferase A1-1 hybrid[80]	NOS	*Homo sapiens*/*Rattus norvegicus*	5ld0
Histone-lysine *N*-methyltransferase SUV420H2[92]	NOS	*Homo sapiens*	3rq4
JAK2 protein tyrosine kinase[93,94]	NOS	*Homo sapiens*	4fvq, 5ut5
Leucine carboxyl methyltransferase 1	NOS	*Homo sapiens*	3iei
OGG1, DNA hydrolase[95,96]	NOS	*Homo sapiens*	1m3q, 2xhi
PHD finger protein 2[97]	NOS	*Homo sapiens*	3ptr, 3pu3, 3pu8, 3pua
Protein-lysine methyltransferase METTL21C	NOS	*Homo sapiens*	4mtl
Protein-tyrosine kinase 6[98]	NOS	*Homo sapiens*	6cz3
Selenophosphate synthetase 1[99]	NOS	*Homo sapiens*	3fd5
Sprt-like domain-containing protein spartan[100]	NOS	*Homo sapiens*	6mdw, 6mdx
TBC1 domain family member 7	NOS	*Homo sapiens*	3qwl
Ubiquitin-conjugating enzyme E2[101]	NOS	*Homo sapiens*	1j7d
Ubiquitin-conjugating enzyme E2[102]	NOS	*Homo sapiens*	3e46
Ubiquitin-conjugating enzyme E2 D2[103]	NOS	*Homo sapiens*	4v3l
Ubiquitin-conjugating enzyme E2[104]	NOS	*Homo sapiens*	6qhk

damage during X-ray diffraction experiments.[2,11] Indeed it is generally accepted that formation of the NOS bridge requires oxidizing conditions, while synchrotron radiation causes reduction.[9,12] A subsequent analysis of the same enzyme by sulfur K-edge X-ray absorption spectroscopy (XAS) conclusively showed the presence of a NOS bridge in solution.[20]

Analysis of crystal structures[12–15] in the Acharya group were unable to definitively differentiate between an oxygen or methylene bridge, as these crystals did not diffract beyond 1.0 Å resolution, which is required to unequivocally determine the bridging atom.[19] However, it was noted that crystals were obtained in non-reducing conditions.[13] The bridging atom is also determinable at lower resolutions (1.1–2.0 Å), however, occupancy and local order impact the reliability of atom identification.[11] There are examples where NOS linkages can be detected by mass spectrometry,[24] although in general it is challenging to detect such a small change (~14 Da compared to reduced enzyme) against background noise and extremely difficult to differentiate a NOS linkage from a methylene bridge (~12 Da) because of the very small difference in mass between these linkages.

NOS bridges have been detected in a large number of other proteins (Table 1) of various types.[11,24,25] A notable example of this is the formation of a NOS bridge within the active site of a mutant New Delhi metallo-β-lactamase-1 (NDM-1) which is Zn^{2+}-deficient. Formation of this NOS bridge is thought to reduce thermal stability of the mutant enzyme compared to the wild-type enzyme by almost 10 °C.[26] The significance of this observation is uncertain since the formation of the NOS bridge is incompatible with the presence of a metal-binding His residue. A novel SONOS extended covalent bond comprising of two Cys residues, a Lys residue and bridging oxygen atoms was also detected in several proteins[11] including the SARS-CoV-2 virus main protease (M^{pro}) and related polyprotein sequences.[27] The residues involved (Cys-22, Cys-44, and Lys-61) are conserved in the main proteases from a wide variety of coronaviruses, including Bat virus, Pangolin virus, and Middle East respiratory syndrome (MERS) virus.[27]

A variety of different types of intramolecular and intermolecular bridges have been reported[11] (Fig. 2, **6** to **10**). The vast majority of these bridges are intramolecular, and occur between residues which are ten or less amino acids apart in the protein primary sequence.[11] Intermolecular bridges are less frequently observed.[11]

Mechanism of NOS bridge formation

The mechanism of NOS and SONOS bridge (Fig. 2) formation has been extensively investigated.[2,27,105] It is generally accepted

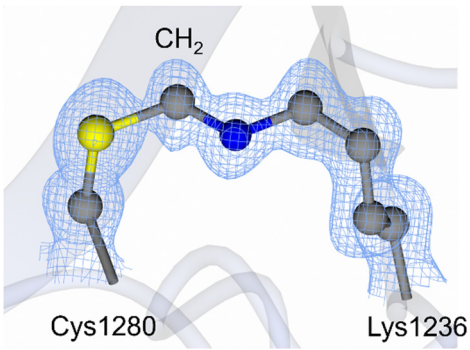
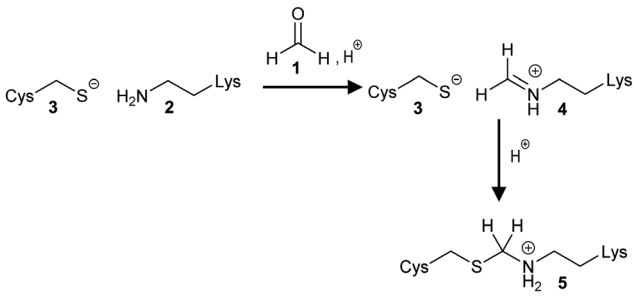


Fig. 1 Methylene bridge reported in the 1.15 Å crystal structure of *Clostridium botulinum* neurotoxin (BoNT) subtype A5 cell binding domain (PDB code 6TWP).[15] The sulfur atom is shown in yellow, carbon atoms in grey, and nitrogen in blue. The 2Fo-Fc electron density map (blue mesh) is contoured to 1σ. In the NOS linkage, the CH_2 group is substituted with an oxygen atom.



Scheme 1 Formation of the methylene bridge **5** using methanal **1**. The Mannich reaction proceeds *via* formation of an imine intermediate **4**.

that formation is an oxidative process. The oxidizing species has not been conclusively identified, but likely to be molecular oxygen[2,17] or a reactive oxygen species.[11,105] Formation of bridges in the presence of molecular oxygen and hydrogen peroxide has been observed.[2] Bridge formation is also reversed in the presence of reducing agents in many cases,[2] although there are examples where bridges are not reduced in the presence of 1 mM DTT but were in the presence of 5 mM DTT.[27]

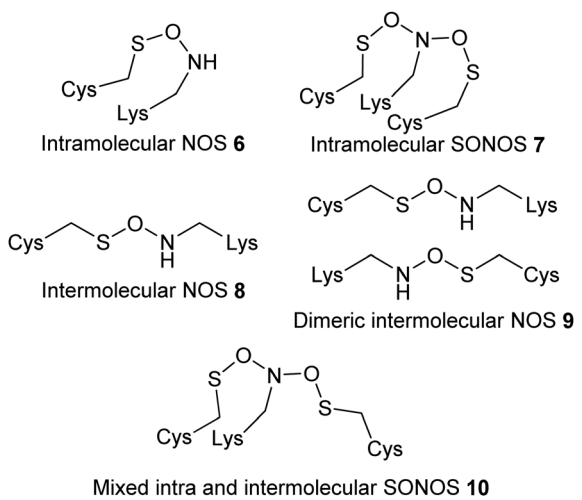
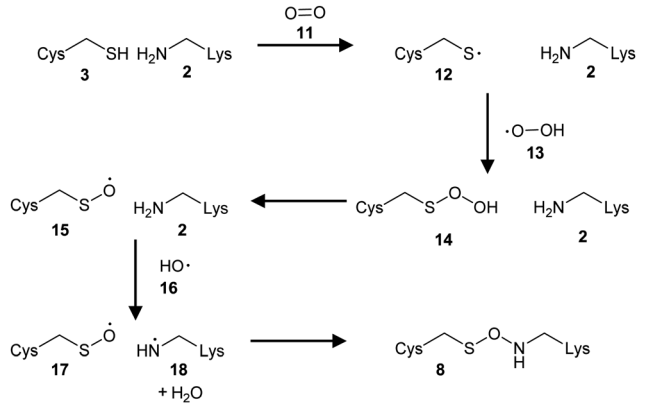


Fig. 2 Different types of covalent bonds observed in proteins.[11]



Scheme 2 Formation of the NOS linkage in the presence of oxygen.[105]

Various mechanisms for formation of the NOS bridge (Scheme 2) have been proposed,[2,17,27,105] including those involving singlet and triplet oxygen, and various heterolytic and radical mechanisms. Theoretically, oxidation of the Lys **2** or the Cys **3** could take place as the first step. However, it is generally recognised that it is difficult for amines to be oxidized in aqueous solution because they are often protonated.[10] Hence oxidation of the Cys **3** sulfur atom is generally considered as the most likely first step.[2,27,105] Both heterolytic and radical reactions have been considered as potential mechanisms. Initial calculations favoured a heterolytic mechanism generating ionic species.[2] However, subsequent analysis[105] suggested that this type of mechanism would favour formation of the sulfinic acid rather than a NOS bridge. On the other hand, a radical reaction gives rise to the desired NOS linkage.

Although it was initially suggested that a Glu residue promoted formation of the NOS bridge,[2] it was subsequently noted that the environment in which the bridge exists is highly variable with different surrounding residues.[11,25] It is now generally accepted that acid/base catalysis is not required for bridge formation in most proteins.[11]

It appears that formation of the SONOS bridge (Fig. 2, 7) in SARS-CoV-2 main protease, which contains two Cys residues bridged to a central Lys residue, is asymmetrical.[105] Formation of a bridge between Cys-22 and Lys-61 is thermodynamically more favoured than is formation of the alternative NOS intermediate where Cys-44 reacts with Lys-61. A similar mechanism to NOS linkage formation has been proposed.[105] However, the presence of two Cys residues mean that formation of a disulfide bond could potentially compete for SONOS formation.[35] The Cys **3** to Lys **2** distance is around 2.6 to 2.7 Å in the NOS linkage,[11] which compares to 2.18 Å and 2.05 Å for reversible and structural disulfide bonds, respectively.[106] It thus appears that some proteins may interconvert between a NOS bridge, a disulfide bond, or the reduced form depending on conditions.[2,12,14,17,35] A similar situation was observed with NOS linkages in BoNT structures.[13]

Although it is generally accepted that NOS and SONOS bridge formation is reversible under reducing conditions,[2,27,105] there have been no reported mechanistic or computational studies on the reduction of NOS bridges to Cys and Lys residues. It is therefore not clear whether the reductive process is a direct reversal of NOS formation or if a different mechanism operates.[17] It is noted that bridge formation is thermodynamically difficult but reduction is easy.[17]

Implications of NOS bridge formation in protein structures

Formation of NOS or SONOS bridges can have a wide variety of effect on the protein. For example, formation of the NOS bridge results in allosteric conformational changes.[2,13] In the cell binding domain of BoNT serotype A2,[12] slight changes in a β-hairpin were observed on going from the reduced to NOS bridged forms. In other cases it can result in subtle allosteric changes resulting in greater protein stability, as measured by resistance to heat-denaturation.[2] In enzymes there is also the potential for modulation of catalytic activity. *N. gonorrhoeae* transaldolase[2] has been shown to be substantially inactivated by NOS bridge formation. Catalytic activity of the wild-type enzyme was diminished by a factor of ~50-fold (as judged by k_{cat}/K_m) in the oxidized form. On the other hand, the enzyme from *N. meningitidis* was ~4.5-fold more active than the *N. gonorrhoeae* enzyme, and activity was diminished to a much lesser extent upon NOS bridge formation (~3.5-fold). Reversal of this inactivation was achieved using reducing agents (DTT or TCEP).[2] Similar observations were made with SARS-CoV-2 main protease (M^{pro}), which is slowly inactivated under non-reducing conditions.[35] The enzyme treated with H_2O_2 at moderate concentrations is fully reactivated upon treatment with DTT. However, treatment of enzyme with high H_2O_2 concentrations results in irreversible inactivation. It is also notable that the enzyme undergoes a dimer to monomer transition upon treatment with H_2O_2, and results in reduced protein stability to thermal denaturation. How generally applicable these phenomena will be is difficult to tell, given that intracellular enzymes usually exist in reducing environments which disfavours NOS bridge formation. There has been discussion about NOS bridge formation as part of a generalized redox response,[11,25] oxidative stress response,[11] and there is some suggestion that NOS or SONOS bridges may also function as oxygen sensors. It is notable that BoNTs eventually end up in cell endosomes, which is an oxidizing environment,[107] implying that NOS formation could be pathologically relevant.[12–14]

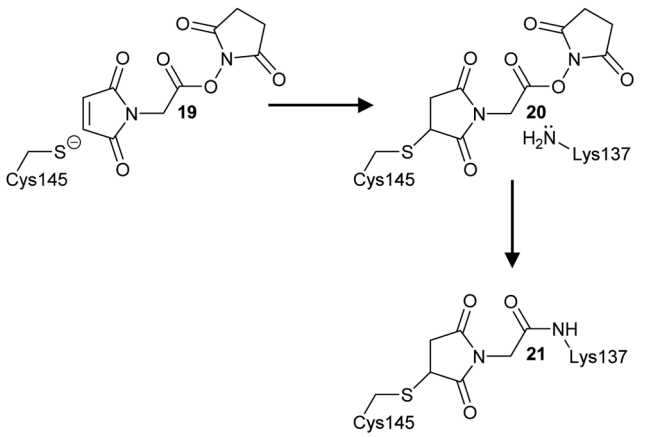
Exploiting protein NOS linkages for drug design

The demonstration that protein NOS linkages can allosterically modulate the activity of enzymes[2,35] opens a new avenue of drug discovery research.[2,35] Allosteric drugs have a number of advantages, including the potential for fewer side-effects due to higher selectivity,[108] acting with naturally occurring ligands rather than having their effects diminished by the ligand,[108] and the ability to drug "undruggable" targets.[109] Covalent drugs[108,110] modifying the Cys **3** or Lys **2** residue would be particularly useful for targets where NOS bridge formation were activating, although there are currently no examples of this. On the other hand, examples where NOS bridge formation is inhibitory[2] may be more difficult to target as this requires that bridge formation (oxidation) is enhanced. Allosteric drugs[11] may lend themselves to this approach by enhancing the required protein conformation for NOS bridge formation. A challenge with this approach is the ability to reliably detect NOS bridge formation in the presence of the drug by crystallographic or other biophysical methods. There is also likely to be a complex interplay between NOS bridge formation, inhibitor potency, and cellular redox environment.

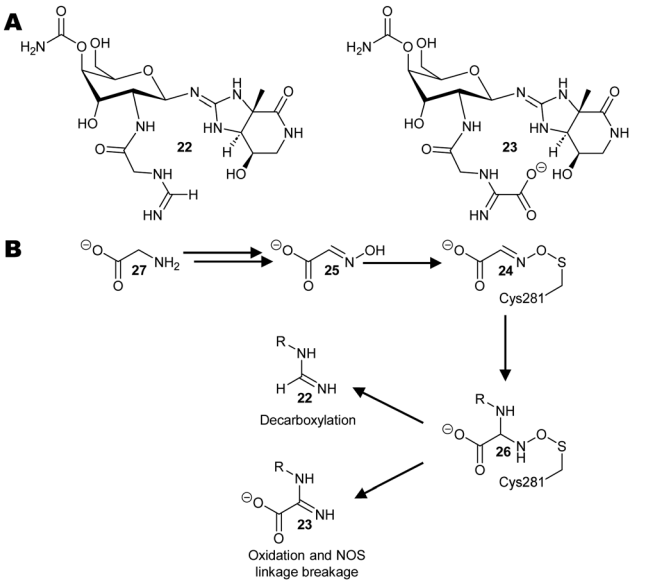
A recent study on the SARS-CoV-2 main protease (M^{pro})[35] provides proof of concept (Scheme 3). Treatment of M^{pro} with the heterobifunctional cross-linker maleimidoacetic acid *N*-hydroxysuccinimide ester **19** inhibited the enzyme ($IC_{50} = 18 \pm 1.8$ μM) and caused dimer to monomer conversion, consistent with SONOS bridge formation. Further analysis demonstrated that the derivatized Cys **3** and Lys **2** residues were not those involved in the SONOS linkage, and that Cys-145 (the catalytic cysteine) was derivatized. Nevertheless, this demonstrates that cross-linking of Cys **3** and Lys **2** residues can be achieved.

NOS intermediates in natural product biosynthesis

A recent paper on streptothricin **22** and **23** antibiotic biosynthesis by Wang *et al.*, revealed a NOS linkage **24** between a catalytic Cys residue and a hydroxylamine small-molecule intermediate **25** was formed during enzyme catalysis (Scheme 4).[111] This *N*-formimidoyl fortimicin A synthase enzyme requires flavin adenine dinucleotide to perform this redox reaction. Examination of the enzymes crystal structure[111] confirmed the presence of a NOS linkage **25**, with geometry and bond lengths like those observed between Cys **3** and Lys **2** residues. Decarboxylation of



Scheme 3 Cross-linking of Cys145 and Lys137 residues in SARS-CoV-2 main protease by maleimidoacetic acid *N*-hydroxysuccinimide **19** to form adducts **20** and **21**.



Scheme 4 Biosynthesis of streptothricin antibiotics from glycine **27**.[111] (A) Structures of representative streptothricin antibiotics **22** and **23**. (B) Putative reaction pathway *via* the NOS linked intermediate **24** observed by X-ray crystallography.

intermediate **26** to give the formimidoylated product **22** readily cleaves the NOS linkage. In contrast, formation of the *N*-iminoacetylated product **23** is less well defined since the proposed mechanism requires oxidation of the side-chain and concomitant reduction of the NOS linkage.

Similar enzymes are found in many *Streptomyces* and other Gram-positive bacteria, and NOS intermediates may play a role in the biosynthesis of complex antibiotics which have similar structures to streptothricins or other natural products where a reactive intermediate needs to be tethered in the enzyme active site.

Conclusions

The discovery of the NOS linkage revealed a new protein post-translation modification which has been shown to be widely distributed amongst proteins.[8,11,17,27,105] A mechanism for its formation has been proposed,[2,27,105] as have a number of roles in reactive oxygen species biology.[25,105] The NOS linkage and the branched SONOS linkage[27] may be as widespread within proteins as disulfide bonds. There are still many questions to be answered, including if NOS or SONOS linkages regulate the activity of many different types of proteins,[25] if they modulate protein turnover,[9] whether the linkage interacts with small molecules such as glutathione or amines,[9,25] and what role these linkages play in disease?

The reduction of NOS and SONOS linkages in cells is completely unexplored. Although promiscuous molybdenum-containing enzymes (mitochondrial amidoxime reducing component; mARC) which can reduce N–O bonds have been characterized,[112–115] it is unclear whether they can reduce NOS or SONOS linkages. All the currently known enzymes are all located within mitochondria. Reduction of N–O bonds has been demonstrated for a wide variety of small molecules. The location of these linkages within substrate proteins would be important. An internal linkage implies that reduction requires either quite large conformational changes or reversible protein unfolding to allow the reductase access. The mARC proteins require NADH and auxiliary proteins to catalyse their reduction, such as cytochrome b5 and cytochrome b5 reductase.[113–115] Intriguingly, in plants the enzyme nitrite reductase (which catalyses the reduction of nitrite to nitric oxide) has also been shown to interact with mARC proteins, implying a potential link with cellular signalling.[113,114]

Abbreviations

BoNT	*Botulinum* neurotoxins
dTDP	deoxythymidine diphosphate
DTT	dithiothreitol
mARC	mitochondrial amidoxime reducing component
NDM-1	New Delhi metallo-β-lactamase-1
NOS	nitrogen–oxygen–sulfur linkage
SARS-CoV-2	severe-acute-respiratory-syndrome-related coronavirus
SONOS	sulfur–oxygen–nitrogen–oxygen–sulfur linkage
TCEP	tris(carboxyethyl) phosphine

Author contributions

MDL wrote the first draft. All authors reviewed the draft, were involved in revision of the manuscript, and approved the final version.

Data availability

No primary research results, software or code are included, and no new data were generated or analysed.

Conflicts of interest

There are no conflicts of interest to declare.

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