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REVIEW

Unusual post-translational protein modifications: The benefits of sophistication

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The proteome of an organism represents the work force that is responsible for cellular activities, regulation and survival. Subsequent to synthesis and folding, there is growing evidence that proteins can undergo several novel and previously unknown post-translational modifications (PTMs) that are structurally and functionally significant. Non-disulphide backbone – side chain or side chain – side chain covalent bonds and supplementary modifications that chiefly generate catalytic centres and render proteinaceous enzymes functionally autonomous, are highlighted in this review. Currently known biosynthetic mechanisms derived using modern methodology for identification of such PTMs are discussed.

Introduction

The work force, responsible for cellular activities, regulation and survival of any organism, is its proteome. Unlike nucleic acids, the innumerable permutations possible with the proteinogenic amino acids confer proteins with a variety of assorted and discrete functions that earmark the diversity among living organisms. Once synthesized, folding of the protein to its optimal two- and three-dimensional structure (with varied levels of order or disorder), and possibly oligomerization, is required for its downstream activity, which may be enzymatic, structural or regulatory. During or subsequent to the process of synthesis, a protein molecule undergoes a variety of post-translational modifications (PTMs) with varied levels of complexity, which are vital for structure and downstream enzymatic, structural or regulatory activity. Disulphide bond formation is the most common and widely prevalent, of all known PTMs.

The six major classes of enzymes can facilely participate in five broad classes of reactions, of which oxidation-reduction reactions and group transfer processes vastly require the presence of small molecules called cofactors that act as the enzyme's chemical teeth. Cofactors (or coenzymes, if they are organic molecules) vary in their physico-chemical properties, ranging from metal ions and organic molecules to prosthetic groups such as porphyrins and vitamins. Standard textbooks define cofactors (or coenzymes) as small molecules that bind to protein active sites and aid in catalysis. Cofactors can be separated from the protein molecule to give rise to the inactive apoenzyme form of the protein. The presence of cofactors for active functioning of the enzyme was for a long time considered so mandatory that when histidine decarboxylase from *Lactobacillus* sp. was found, in the early 90s, to function in the absence of PLP (pyridoxyl phosphate, the normal cofactor used), it came in as a surprise for the scientific community. The crystal structure of this unusual enzyme, published in 1993, for the first time revealed an unusual modification that efficiently functions as an analogue of the normal cofactor, thereby completely dispensing the need for PLP.[1] The cofactor, generated by autocatalytic serinolysis of Ser 82 in the proenzyme, forms a Schiff base with the substrate and facilitates histidine decarboxylation.

Over the last decade, a large number of unusual PTMs in proteins have been unearthed, which includes newly identified cross-links between amino acid side chains, thanks to high-resolution structures, combined with biochemical studies. With the advent of modern techniques and methodology for the study of proteins in the molecular level, including mass spectrometry, high resolution X-ray diffraction and nuclear magnetic resonance (NMR), spectroscopic methods, it has now become possible to discover and characterize several novel and previously unknown protein PTMs, and map functional and structural significance to these modifications. The only well-known side chain cross-link in proteins is the disulphide. Identification and characterization of novel, non-disulphide covalent bonds which include carbon-carbon, carbon-sulphur, nitrogen-sulphur, nitrogen-carbon and oxygen-carbon bonds between amino acid side chains at protein active sites has paved way for newer and more stable post-translational modifications in proteins. Such PTMs not only contribute to additional diversity in these biomolecules but also play a key role in many cellular processes catalysed by enzymes.

While changes such as acetylation, glycosylation, lipidation, phosphorylation etc., are well known and commonly observed in several proteins across organisms, this new class of covalent post-translational modifications (PTMs) is increasingly being recognized in a small class of proteins. This unique class of PTMs appositely adopted by nature includes non-disulphide peptide backbone – side chain or side chain – side chain covalent bonds that chiefly generate catalytic centres and render such proteinaceous enzymes functionally autonomous, and thereby independent of external organic cofactors. These adept intrinsic cofactors are primarily derived from and composed of amino acids like tryptophan, tyrosine, cysteine, histidine and lysine.

This review attempts to summarize such unique PTMs, probe their possible function and discuss the implications of such modifications to the design of artificial enzymes. Proteins bearing such unusual covalent modifications are largely enzymes, but also include a smaller, yet significant, class of structural proteins, which we categorize into the following sections:

1. Proteins that contain side chain – side chain cross-link, and are a broader class of enzymes, include oxidases, peroxidases, catalases etc. These cross-links are observed between those side chains of amino acids present near the active site, and are usually coordinated with a metal ion. These cross-links either provide structural stability to the active site and/or participate in the catalytic reaction itself. Some examples we discuss include cytochrome c oxidase, catechol oxidase, catalase peroxidase and galactose oxidase.
2. Enzymes that contain side chain – side chain, side chain – backbone cross-link or other backbone alterations with supplementary modifications include dehydrogenase, oxidases etc. These cofactors are amino acid – derived quinones that are present at the active site and provide structural stability to the enzymes' active site and participate in the catalytic activity. Some examples we discuss include lysyl oxidase, methanol dehydrogenase and a few amine dehydrogenases.
3. A small class of non-enzymatic proteins, which contain unusual covalent non-disulphide cross-links, provides special characteristics and inter subunit structural stability to the molecule. Examples for this class include ranasmurfin, green fluorescence protein (GFP) and collagen. Such modifications are not just confined to larger proteins and enzymes, but are also detected in small, ribosomally encoded peptides, examples for which include few Amanita toxins and lantibiotics.

In this framework, we review the biochemical pathways by which such unusual post-translational modifications are synthesized, the structures and local conformational geometry of such modifications, the functional implications and the recent advancements in the field, which are increasingly fascinating to examine. While we have tried to include exciting findings and known examples of unusual PTMs, there may still be a few modifications that have escaped our notice, or have already been covered extensively elsewhere,[2] and are therefore not dealt with in detail herein.

Side chain cross-links in enzymes

Enzymes catalyse nearly all known biochemical pathways within any living organism, and are vital for sustenance of life forms. Many enzymes are assisted by cofactors for their functioning, which may range from inorganic metal ions, organic molecules (vitamins, nucleosides etc.) to molecules that are nearly 1 kDa in molecular weight. It is known that some enzymes require multiple cofactors; for instance, pyruvate dehydrogenase utilizes five cofactors and a metal ion.[3] Addition of such extrinsic substrates to enzymes renders it functional. Recently, however, with the advent of advanced biochemical and biophysical methods, a new class of PTMs which result in amino acid derived cofactors were discovered.[2c] Such cofactors are of great biological significance, as these structures help expand the range of chemical reactions involving amino acids, as well as they create new structural motifs that provide structural stability to the active site.

Oxidases

Galactose oxidase. Galactose oxidase (GO) is an enzyme which catalyses the oxidation of primary alcohols to their corresponding aldehydes by reducing molecular oxygen to hydrogen peroxide. The reaction involves a transfer of dihydrogen between the substrates.

GO is a copper metalloenzyme which contains an unusual tyrosine-cysteine thio-ether cross-link in its active site. This cross-link serves as an intrinsic cofactor which helps in transferring the dihydrogen, unlike other enzymes which employ extrinsic cofactors like flavins, nicotinamides and quinones. The active site is a copper complex in which the copper is surrounded by Tyr 272, Tyr 495, His 496 and His 581. Tyr 272 C^{ε} is cross-linked to the side chain sulphur of Cys 228 (residue numbering is of GO from *Fusarium* sp.) (Figure 1).[4]

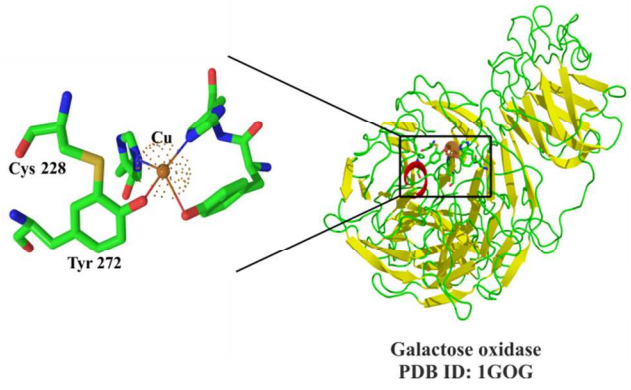

Figure 1. Ribbon diagram of galactose oxidase, highlighting the Cys-Tyr cross-link.[4]

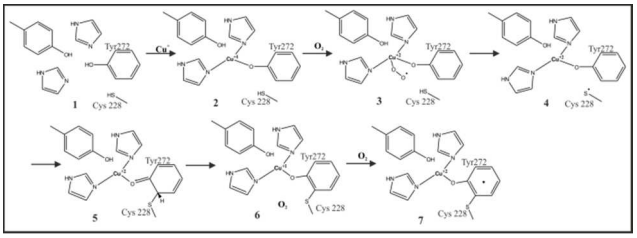

Figure 2. Proposed mechanism of galctose oxidase Tyr-Cys cofactor. Figure re-drawn with permission from reference [2f].

The biogenesis of the cofactor is only dependent on the presence of Cu^{1+} and molecular oxygen.[4a] Kinetics data comparing the cofactor synthesis in the presence of Cu^{1+} and Cu^{2+} supports the sole requirement of monovalent copper (since the divalent form is generated during the reaction, as described below). In this process, Cu^{1+} initially binds to a pre-organized active site, and coordinates with tyrosine, forming a reduced metal complex. O_2 reacts directly with the reduced metal complex to form a Cu^{2+} – superoxide, which is a reactive adduct. This oxygenated adduct abstracts a hydrogen atom from the active site Cys 228, resulting in the formation of a thiyl radical, which adds to the Tyr 272 aromatic ring. The addition of this thiyl radical to tyrosine gives rise to a non-conjugated system in the phenolic side chain. The Tyr aromaticity is then restored by deprotonation and reduction of Cu^{2+} to Cu^{1+}.[2f] Hence, during the reaction turnover, this Cu^{1+} form at the active site corresponds to the reduced form of the protein. This reacts further with O_2. The oxidised Cu – tyrosyl radical complex is subsequently formed with the action of O_2.[2f] The reaction is presented in Figure 2.

The Tyr-Cys thio-ether bond influences the stability, reduction potential and catalytic efficiency of the GO active site. It has been reported that the lack of the linkage has only a minimal effect on the Cu coordination at the active site. However, it influences the π-stacking interaction between the Cu bound Tyr 272, and a nearby Trp 290, which is important to keep the active site geometry intact. This is evident by comparison of the active site geometries of the precursor and mature protein, available from crystal structures of GO from *Fusarium* sp.[4]

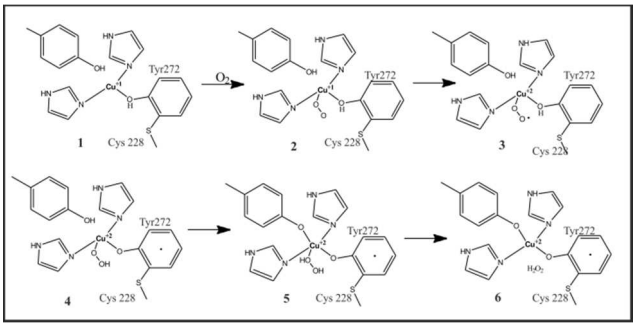

Figure 3. Dioxygen reduction mechanism of galactose oxidase. Figure re-drawn with permission from reference [2f].

Furthermore, the cross-link acts as an independent redox cofactor, allowing easy electron delocalisation. The Cu(II)/Cys-Tyr* cofactor carries out a $2e^-$ oxidation of primary alcohols to aldehydes via radical mechanism.[4a] The cross-link also reduces the reduction potential by about 75 mV, which is sufficient to modulate the reactivity so that the activated C-H bond of the alcohol is attacked preferentially by the tyrosine.[2h] The mechanism by which the molecular oxygen is reduced to hydrogen peroxide is shown in Figure 3.[2e, 2f] The protein shows activity only in the mature cross-linked form.

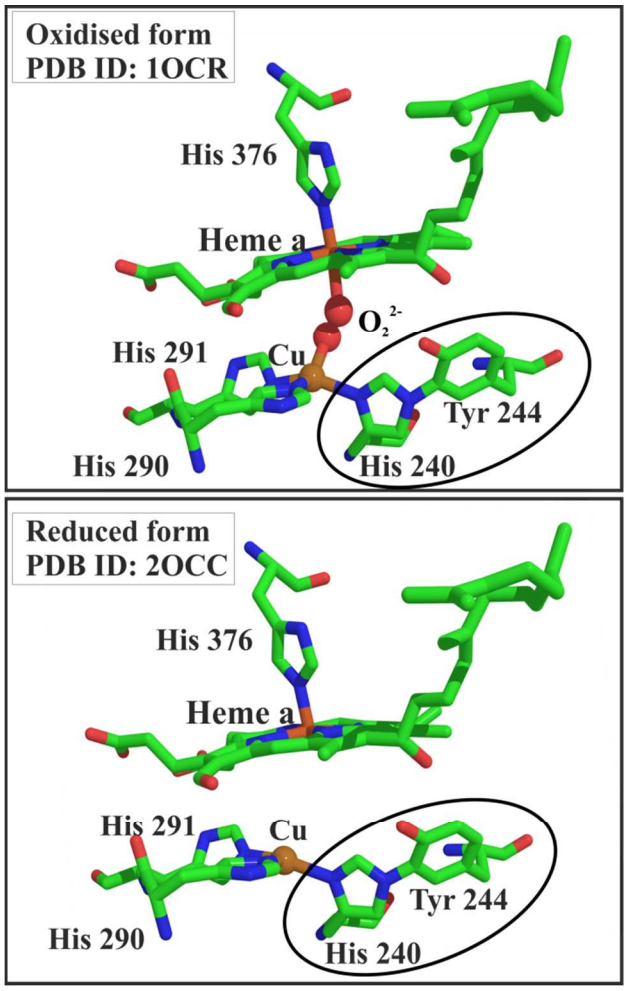

Figure 4. Ribbon diagram of cytochrome c oxidase (right), which harbours the His-Tyr cross link at the oxygen binding site. Shown as stick representations (left) are the oxidised and reduced forms of the active site, highlighting the importance of the cross-link in binding molecular oxygen.[5]

Cytochrome c oxidase. Cytochrome c oxidase is an enzyme, which forms a part of the electron transport chain complex and a component of inner membrane of mitochondria in eukaryotes and plasma membrane in prokaryotes. It is the final complex in the respiratory electron transport of the aerobically respiring cells.[6] It catalyses the reaction of O_2 to H_2O, by coupling with the reaction of proton pumping across the membrane, and

providing the potential energy (stored in the proton gradient thus formed) needed for ATP production.[7] The subunit I of the enzyme contains a heme Fe-a_3-CuB binuclear catalytic centre. This catalytic centre is coordinated with six histidines.[8] One of these histidines is involved in an unusual cross-link through its N^{ε} to the $C^{\varepsilon 2}$ of Tyr (Figure 4). Crystal structures of this protein from bovine heart as well as *Paracoccus denitrificans* confirm the presence of this cross-link.[5, 7] In *P. denitrificans*, this conjugated ring system is formed between His 240 and Tyr 244.[5, 7] Nothing is known about the mechanism of formation of the bond during the post-translational modification.

One of the reactions catalysed at the active centre of cytochrome c oxidase, is dioxygen activation. It is both important, yet equally difficult, to transfer electrons and protons successively to dioxygen (O_2), while ensuring no formation or release of the superoxide anion radical $\bullet O^{2-}$, peroxide O_2^{2-} and the hydroxyl radical •OH, during the peroxide split, since all these molecules are deleterious to the cell. The His-Tyr linker plays a very important role in achieving this. Further, it is believed that the pK of the hydroxyl group of Tyr is significantly lowered, than free Tyr, due to the cross-link, which is important for Tyr to act as a proton donor in reduction of molecular oxygen by the enzyme.[7] The mechanism which has been proposed for the peroxide split is described in detail elsewhere.[8]

Using chemical model studies, it has also been shown that the His-Tyr link has a role in the proton pumping action of the enzyme and plays a role in gating the K and D channels.[9] The K and D channels present in the protein facilitate the intra-protein proton transfer by connecting the active site where the dioxygen is reduced, to the internal aqueous phase. The His-Tyr link therefore acts as an electron/proton acceptor/donor and helps in the K and D channel gating.

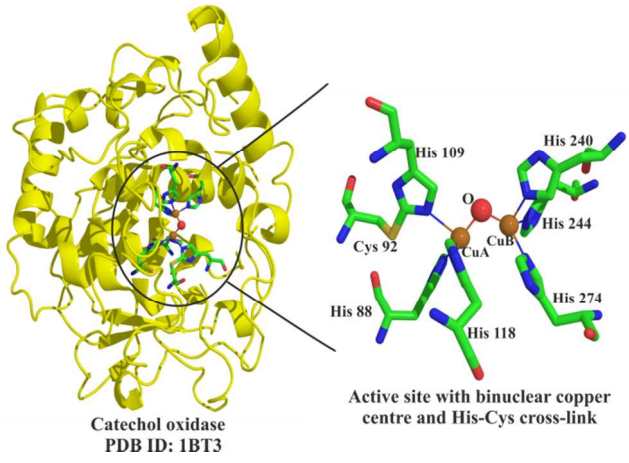

Figure 5. The unusual Cys-His cross-link in sweet potato catechol oxidase (shown on the left as ribbon diagram) is highlighted on the right.[10] The active site contains two copper atoms (CuA and CuB) that are coordinated by three histidines each.

Catechol oxidase. Catechol oxidase, also known as *o*-diphenol oxidase, is a member of the Cu-3 family, which catalyses the oxidation of catechols to *o*-quinones.[11] At the active site of the enzyme, it contains a dinuclear copper centre. Each copper atom is coordinated by three histidine residues; in the catechol oxidase from *Ipomoea batatas*, His 88, His 109 and His 118 coordinate with the CuA and His 240, His 244, His 274 coordinate with CuB (Figure 5).[10] Interestingly, the C^{ε} of His 109, which is a ligand for the copper is linked to the sulphur of Cys 92 by a covalent linkage. It has been suggested that during catalysis the bond stabilizes the tyrosine radical generated.[11] This covalent bond has some structural importance as well. The bond helps in creating structural restraints on the ligand sphere of CuA in the resting enzyme form, resulting in an unusually distorted trigonal bipyramidal geometry on CuII. Here, the electronic state of the metal is optimized for oxidation of *o*-diphenol, and in the redox processes, allows rapid electron transfer. The proposed mechanism of cofactor action in the enzymatic reaction is schematically represented elsewhere.[12] The displacement of the His 109 and any bidentate binding mode of the substrate are prevented by this His-Tyr linkage.[10, 12]

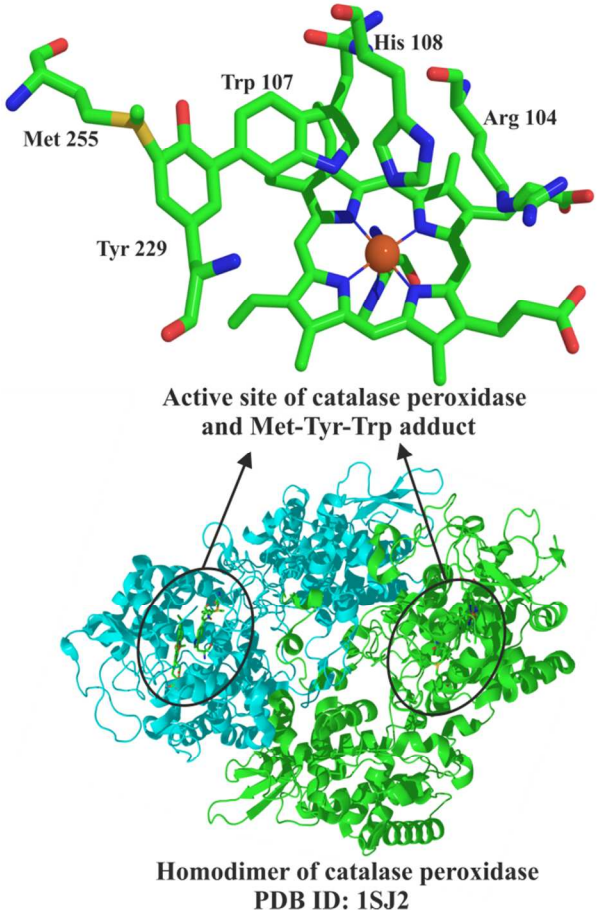

Figure 6. Ribbon diagram of catalase peroxidase highlighting the location of the cross-link and the active site, shown in stick representation.[13]

Peroxidases

Catalase peroxidase. Catalase peroxidase (KatG) is a bifunctional heme dependent enzyme present in bacteria, archea bacteria and some fungi. KatG plays an important role in activating the anti-tubercular prodrug isoniazid. KatG carries out both peroxidase and catalase activities.[14] An amino acid adduct, which is formed by the post translational modification of three distal amino acids, is important for its catalase activity. In KatG of *Mycobacterium tuberculosis* Tyr 229 is linked at its *ortho* position to Met 255 and Trp 107 (MYW).[2g, 13] The proposed mechanism for the formation of the adduct is described in great detail elsewhere.[14b] It has been reported that a tyrosyl-like radical is formed during the reaction of the enzyme with H_2O_2, and persists for the period of turnover of excess peroxide. By site directed mutagenesis, EPR studies and DFT calculations, it has been shown that Tyr 229, along with the entire MYW adduct, is critical for the catalase activity of the enzyme and also for maintaining the heme cofactor in a catalytically competent state for both the enzymatic activities.[2g] The structure of the adduct is illustrated in Figure 6. Formation of the adduct, and its role in the catalase activity of the enzyme is shown in Figure 7.[2g]

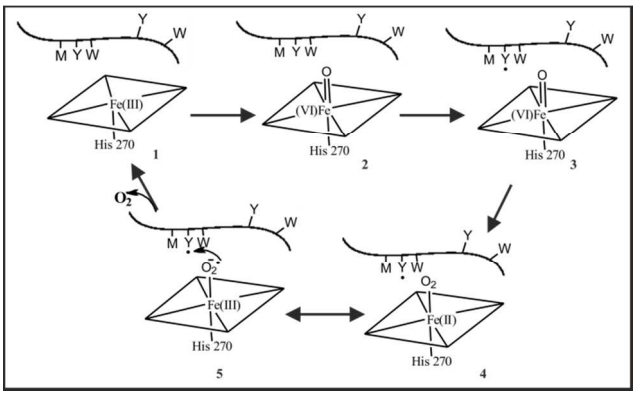

Figure 7. Proposed role of the MYW adduct in the catalytic activity of catalase peroxidase. Figure adapted with permission from [2g]. Copyright (2010) American Chemical Society.

Cytochrome c peroxidase (CcP). The ferrocytochrome c – hydrogen peroxide – oxidoreductase, or cytochrome c peroxidase (CcP, in short), is a 32 kDa mitochondrial intermembrane space protein present in various organisms. It mainly functions to protect against high concentrations of H_2O_2, generated as a process of the mitochondrial electron transport chain. The reaction involves His 52 (numbering from *Saccharomyces cerevisiae*), which functions as a general acid-base catalyst.

A study that attempted to generate mutational variants of CcP, in attempts to engineer novel enzymatic activity into CcP, replaced the distal His 52 with Tyr.[15] The protein was crystallized in the $P2_12_12_1$ space group and solved to a 1.65 Å resolution.[15] Surprisingly, the bond distance obtained between Tyr 52 $C^{\varepsilon 1}$ and the neighbouring Trp 51 $N^{\varepsilon 1}$ was found to be

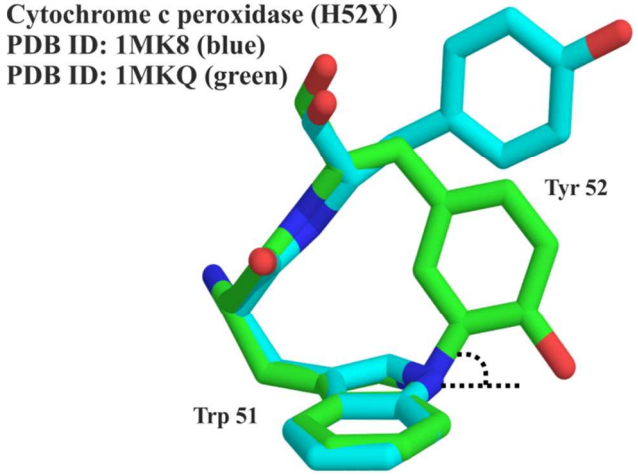

Figure 8. Superposition of the crystal structures of cytochrome c peroxidase highlighting the residues undergoing cross-link. In the absence of a strong oxidising environment, the cross-link is not observed (blue). In the presence of iron and peroxide, the cross-link is formed. The Trp $N^{\varepsilon 1}$ – Tyr $C^{\varepsilon 1}$ bond is bent ~ 64° from the aromatic plane.[15]

<1.6 Å, which, after structure refinement, was seen to have a bond distance of 1.48 Å, clearly indicating a covalent link between Tyr and Trp side chains. Since CcP was crystallized along with the redox active Fe^{3+}-porphyrin, a crystal structure solved using the redox inactive Zn^{2+}-porphyrin not only lacked the cross-link but also positioned Tyr 52 ~ 60° away from the active site.[15]

The unique Tyr-Trp cross-link was therefore established as a crystallization artefact, formed as a result of an old batch of 2-methyl-2,4-pentanediol (MPD) used in the crystallization set-up (MPD generates breakdown products such as peroxides). Using an elegant combination of conditions involving zinc or iron, and treatment with H_2O_2, Bhaskar *et al.* demonstrated that both iron and peroxide are important for the cross-linking process.[15] Since aromatic radicals are inert to nucleophilic attack, formation of the cross-link mandates concurrent oxidation at both sites, and is likely to occur via a peroxidic intermediate.

Interestingly, the cross-link results in a highly pyramidalized intermediate, with the Trp $N^{\varepsilon 1}$ – Tyr $C^{\varepsilon 1}$ bond bent ~64° from the aromatic plane (Figure 8). A similar observation has been made earlier only in the case of the Rebeccamycin class of indolocarbazole glycosides, where the indolic N – sugar bond is ~27° out of the aromatic plane.[16] Semi-empirical calculations carried out suggested a small energy cost of ~2 kcal/mol for a 30° deformation, but an immense 9.8 kcal/mol for the observed 60° deformation in CcP.[15] While the implications of this modification *in vivo* is still unclear, CcP may serve as an excellent model system for studies on unusual cross-links in proteins, and their associated stabilization in a three-dimensional protein scaffold. Further, this experimental artefact serves as the only known example of a Tyr-Trp cross-link in proteins.

Catalases

Catalase HP II. HP II is a catalase found in all aerobic organisms, and it degrades hydrogen peroxide to oxygen and water. It has been shown that the enzyme's active site exists in two forms: one with a heme b and other with heme d. The heme b is oxidised to heme d by the catalase itself, during the catalysis reaction.[17] A novel covalent bond, shown in Figure 9, was identified in the enzyme between the Cβ of Tyr 415 and N^{δ} of His 392 (numbering from *E. coli*). Mutant forms of HP II, which lack this linkage or have a mutation at His 392, do not catalyse the reaction of heme conversion, clearly suggesting that this linkage has a role in the conversion of heme.[18]

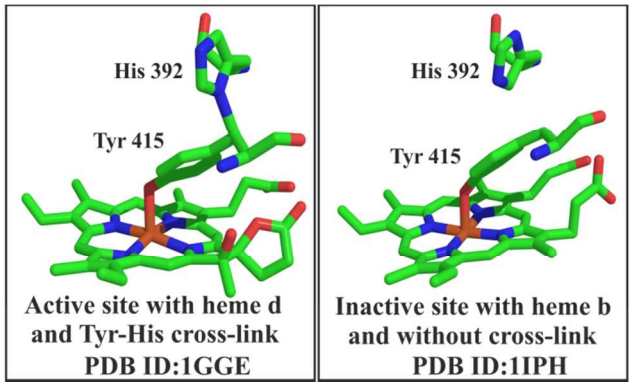

Figure 9. Each subunit of the homotetramer of catalase HP II harbours the Cys-His cross link. The active site bears the cross-link (right),[18] whereas, in the absence of the cross-link (left), the protein is inactive.[19]

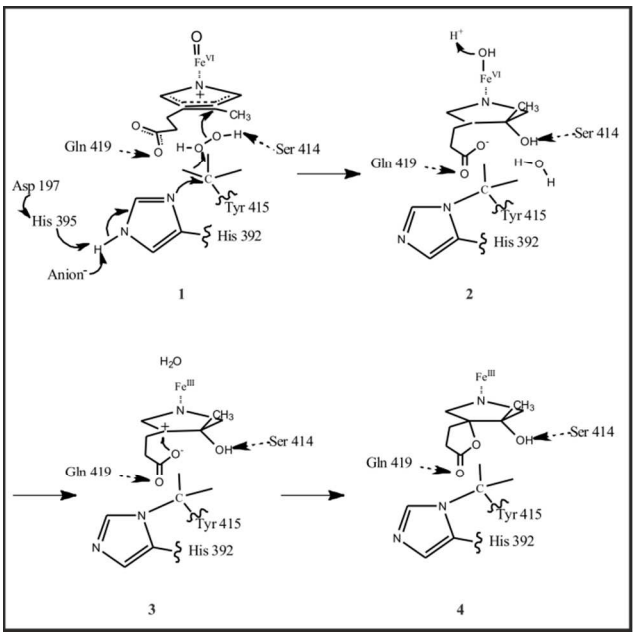

Figure 10. Proposed mechanism of formation of the His-Tyr bond in *E. coli* catalase HP II is shown. Figure re-drawn with permission from reference [19a].

However, this residue is not conserved in all catalases, and is therefore not essential for all members of this family. For example, the catalase from *Proteus mirabilis* possesses a methionine sulphone near the active site.[18]The proposed mechanism for the conversion, although not satisfactory and requires revision based on further experimental analysis, is shown in Figure 10.[19a] The current model shows that the two reactions, the heme conversion and the novel bond formation, are coupled by the catalase. The mutant forms lacking the ability of heme conversion supports this mechanism. The linkage may also add rigidity to the active centre and the movement of the electrons can be favoured by the extended structure.[19]

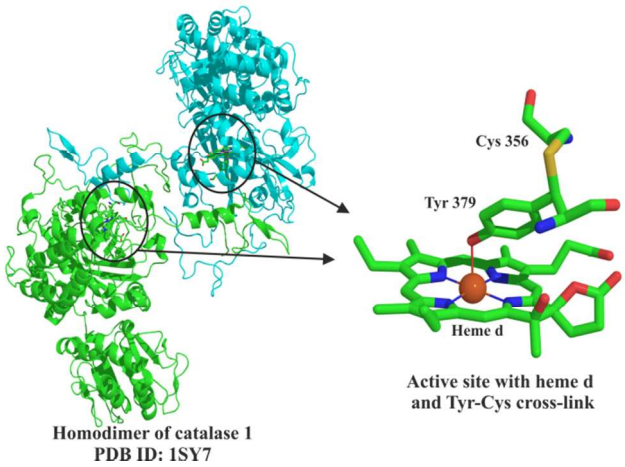

Figure 11. Crystal structure of the catalase I homodimer shown here from *N. crassa*.[20] The unusual Cys 356 – Tyr 379 cross-link formed near the active site is shown on the right.

Catalase I. Catalase I is a large (80 kDa monomer),[21] tetrameric (320 kDa), highly efficient and durable enzyme, which dismutates hydrogen peroxide into one molecule of oxygen and two water molecules. It is active even at molar concentrations of H_2O_2, and is found in non-growing cells of archea, eubacteria, fungi, plants and animals and asexual spores. X-ray crystallographic studies of catalase I from *Neurospora crassa*, and mass spectrometric measurements, have revealed that the enzyme contains an unusual covalent linkage between the sulphur of Cys 356 and C^{β} of Tyr 379 (Figure 11).[20] The possible mechanism for the generation of this covalent linkage is shown elsewhere.[20] The Tyr-Cys covalent bond makes the tyrosine less prone to transfer electrons to compound 1 for the formation of compound 2, which makes the enzyme resistant to the enzyme inhibition and inactivation by high concentrations of H_2O_2.

Oxygenases

Cysteine dioxygenase. Cysteine dioxygenase is an iron-dependent enzyme, which regulates intercellular cysteine levels

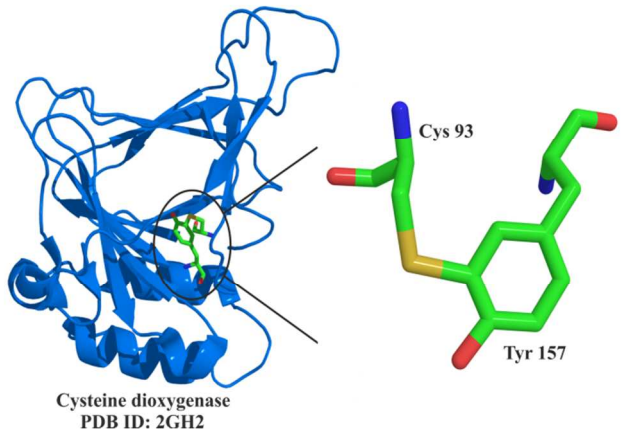

Figure 12. Crystal structure of cysteine dioxygenase, shown here as ribbon diagram, highlighting the thio-ether link between Cys 93 and Tyr 157.[22]

by catalysing the reaction of converting cysteine to pyruvate and cysteine sulfinate. The reaction in itself is important for several metabolic pathways, as it provides inorganic sulphate and pyruvate as metabolic precursors for other biochemical processes. Further, it controls the oxidation and excretion of sulphur from methionine and is essential for taurine biosynthesis. Defects in the activity of the enzyme render an imbalance in the levels of taurine, sulphate and cysteine within the cell.[23] Additionally, defects in cysteine catabolism, which can be induced by blocking this enzyme, lead to depletion of sulphate levels; this was shown to occur in patients suffering from Parkinson disease, Alzheimer's disease, motor neuron disease, rheumatoid arthritis and systemic lupus erythematosus,[22-23] indicating that the active form of cysteine dioxygenase is important to balance sulphur levels and maintain homeostasis.

The enzyme contains a cofactor in its active site, which harbours a thio-ether link between the sulphur of the cysteine and the side chain aromatic carbon of tyrosine (Figure 12).[22] The cofactor is not essential for the catalytic action, but the formation of the cofactor enhances the catalytic action and the catalytic half-life of the enzyme.[24] It is also interesting that the cysteine substrate is important for the cofactor formation. When the cysteine levels are low within the cell, the enzyme is degraded by ubiquitination, which helps in conserving the cysteine levels in the cell. When the cysteine levels are high, the cofactor is formed, which increases the catalytic half-life of the enzyme by nearly 10-fold, and helps in clearing the excess cysteine.[25] Under extreme conditions of cysteine accumulation within the cells, up to 450-fold enhancement in the enzymatic activity is observed as a consequence of this modification, which helps in effective clearing of the excess cysteine. The proposed mechanism for the cofactor synthesis has been deduced by the Stipanuk group.[24] Cysteine dioxygenase is one of the very few examples wherein a non-disulphide reversible post-translational protein modification is observed.

Cross-links with supplementary modifications

Protein PTMs that generate catalytic and redox-active sites usually involve cross-linking coupled with other modifications, primarily side chain oxidation.[2d] For example, intrinsically formed redox cofactors predominantly involve Tyr or Trp side chains. These quinone modifications have been extensively reviewed recently,[26] and are therefore presented here in a succinct manner. Other modifications that involve backbone residues and render the enzyme cofactor independent, are described separately.

Quinones.

Lysyl tyrosine quinone (LTQ). LTQ is the cofactor formed during the post-translational covalent modification between the carbon of an oxidized tyrosine ring and the amide of a lysine side chain.[27] It is prevalent across mammalia, in the protein lysyl oxidase (LOX), and plays an important role in the development and maintenance of the extracellular matrix (ECM); LTQ is critical for LOX functioning. Collagen and elastin in the ECM are oxidized at lysine residues, which lead to the stabilizing covalent cross-link in these fibrous proteins.[28] LOX family proteins also play a role in the epithelial mesenchymal transformation, which is believed to affect cell movement, tumour invasion and metastasis. While these post-translational cross-links are beneficial for added stability of the ECM, over-expression and unusual LOX activity also leads to disorders of the connective and fibrotic tissue,[29] and is reported as being a risk factor for diseases including ovarian cancer.[30]

Tryptophan tryptophylquinone (TTQ). The Kennard's group identified, in 1979, that a pyrrolo quinolone quinone (PQQ) moiety was the redox cofactor of the enzyme methylamine dehydrogenase (MADH).[31] However, the PQQ derivative could never be isolated from the protein molecule. It was only in 1991, that resonance Raman studies, ^{1}H and ^{13}C NMR and DNA-sequence-deduced amino acid sequence of MADH from *Methylobacterium extorquens* AM1 together identified the cofactor of MADH as 2,4'-bitryptophan-6',7'-dione, or simply, tryptophan tryptophylquinone (TTQ).[32]

TTQ formation is a result of post-translational modification of two tryptophan residues, in which one, or both tryptophans may be oxidized. It is a prevalent cofactor in several amine dehydrogenases,[32] For example, in MADH from *P. denitrificans*, Trp 57 undergoes oxidation in two sites on the indole ring, following which it establishes a covalent link to the indole ring of Trp 108. The protein MauG plays an important role in the synthesis of TTQ,[33] as MauG inactivation by site directed mutagenesis adversely affects TTQ formation.[33] MauG particularly participates in the introduction of the second oxygen into the monohydroxylated indole ring of Trp 57 and covalently linking this ring to Trp 108.[34] It appears that MauG and MauL play a concerted role in generating radical intermediates on the pre-MADH substrate. While the cross-linking and oxygen incorporation are directed by the substrate,

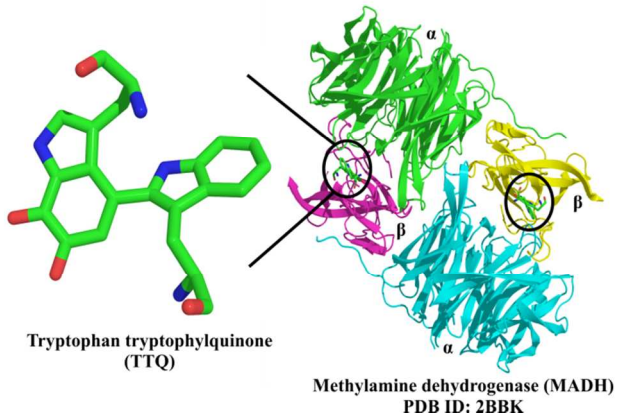

Figure 13. The TTQ cofactor (left) found in the MADH heterodimer (right).[35]

details of the order of events and the mechanism of MADH modification are yet to be deduced.[36]

MADH is a heterodimer of two α-subunits (45 kDa each) and two β-subunits (14 kDa each). The β-subunit houses the TTQ (Figure 13).[35] The cofactor is important for the proper assembly of the α- and β-subunits, in the absence of which dissociation of the subunits occurs. The cofactor is also essential for the redox and catalytic properties of the enzyme, which catalyses the deamination of methylamine to formaldehyde and ammonia (Figure 14).

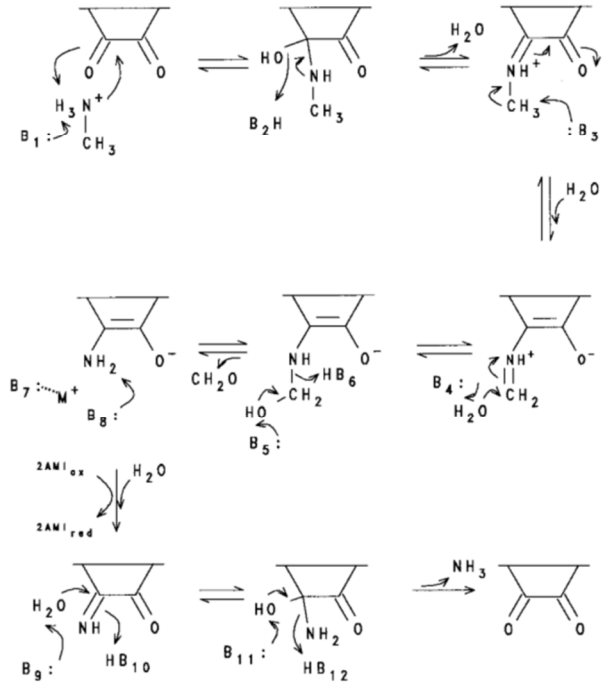

Figure 14. Role of TTQ in the redox reaction catalysed by MADH. Only the quinone region of TTQ is shown, for simplicity of illustration. Figure reproduced with permission from reference [35].

The catalysis proceeds via a reductive reaction, wherein a Schiff base is formed between the amine substrate and C6 of TTQ.[35] Release of the aminoquinone intermediate is accompanied by reduction of the adduct. A general base is required to abstract a proton from the methyl carbon atom, which leads to reduction of the TTQ cofactor. Regeneration of the TTQ cofactor is achieved in the oxidative reaction of the catalysis, wherein two electrons are transferred from a type-I copper protein (amicyanin in the case of MADH), with the release of the ammonia product and the corresponding aldehyde.[37]

Cysteine tryptophylquinone (CTQ). CTQ is one other quinone-based cofactor found in quinohemoprotein amine dehydrogenase (QHNDH), and involves the post-translational oxidation of the indole ring of tryptophan similar to TTQ in MADH. In QHNDH from *Paracoccus denitrificans*, oxidized Trp 43 is covalently linked to the sulphur of Cys 37, giving rise to the CTQ cofactor (2.05 Å crystal structure).[38] *P. denitrificans* QHNDH is a heterotrimeric (αβγ) protein, with three novel cross-links, in addition to the CTQ, found in the γ-subunit.

The *P. denitrificans* QHNDH α-subunit is of 489 residues and is a di-heme c-type cytochrome with four Cys involved in thio-ether linkages to the two heme groups. The β-subunit has a 7-bladed β-propeller motif, folded as a single domain, and is of 337 residues. The γ-subunit is the smallest (82 residues) and is a globular protein with little secondary structure. It is sandwiched between the α- and β-subunits. Despite the presence of four Cys residues, there are no disulphides in this subunit. Stability of this subunit arises from the three unusual cross-links formed by key residues, namely, Cys-Asp, Cys-Glu for structural stability (discussed later), and the cysteine tryptophylquinone (CTQ), shown in Figure 15, which forms a part of the active site.[38-39] These cross-links are formed between the sulphurs of cysteines and either the β- or γ- methylene carbons of aspartic or glutamic acid, and provide structural stability to QHNDH,[38-39] in a manner similar to the inter-subunit disulphides of MADH.

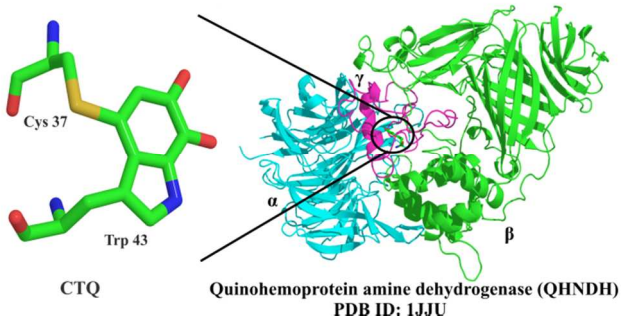

Figure 15. The smallest (γ) subunit of QHNDH (shown in pink) harbours the CTQ cofactor, formed by the covalent cross-linking of a cysteine side chain with the oxidised indole of tryptophan.[38]

The mechanism by which the CTQ modification occurs is poorly understood. It has been shown that the ORF2 protein of *P. denitrificans* (putative [Fe-S]-cluster and (S-Ado-Met; SAM)-binding protein) plays an important role in the posttranslational processing of the γ-subunit.[40] Further, the α-subunit harbours two c-type heme moieties, and it has been speculated that this di-heme subunit may play a role in the biosynthesis of CTQ in a mechanism similar to the role of MauG in TTQ biosynthesis.[26a] It is, however, evident that the QHNDH and radical SAM enzyme are encoded in the same gene region in *P. denitrificans*.

CTQ is important for the catalytic activity of QHNDH. The overall reaction catalysed by QHNDH is similar to MADH (see Figure 14).[41] Unlike MADH however, it is difficult to separate the reductive and oxidative half reactions as the intermediate electron acceptors (the two heme units) are present within the α-subunit of the enzyme. Asp 33 in the γ-subunit serves as the active site base for proton abstraction.[41] The re-oxidized QHNDH is generated when these acceptors donate the electrons to azurin in *Pseudomonas putida*,[38] or cytochrome c550 in *P. denitrificans*.[39, 42]

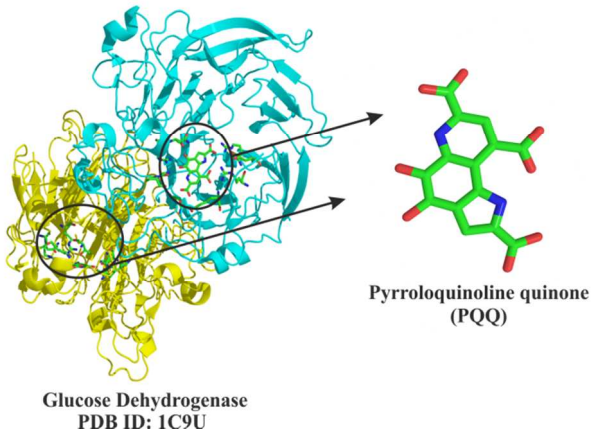

Figure 16. Crystal structure of glucose dehydrogenase (shown as ribbon diagram) revealed the presence of the PQQ cofactor, highlighted here as stick representation.[43]

Pyrroloquinoline quinone (PQQ). PQQ is a redox cofactor in many bacterial dehydrogenases, for example, methanol dehydrogenase and glucose dehydrogenase (Figure 16).[43] It is a water soluble, heat stable tricyclic orthoquinone, which provides unique redox features to these enzymes.[44] PQQ is important for the catalytic activity of those enzymes that catalyse the reaction of cysteamine oxidation to cystamine and the oxidative fermentation of D-glucose to D-gluconate.[45] The catalytic mechanism of glucose dehydrogenase is proposed to occur either via a hemiketal intermediate or hydride transfer.[46] In the mechanism involving the hemiketal, an oxyanion is produced by proton abstraction from the alcohol substrate by a general base (here, it is probably the Asp residue present near the active site). This oxyanion attacks the electrophilic C-5

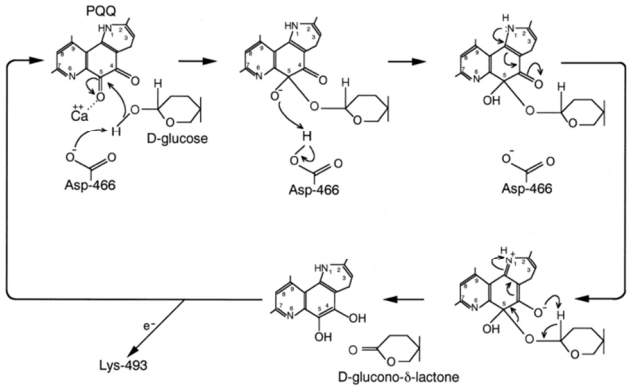

Figure 17. Proposed function of PQQ in catalysis. Figure reproduced with permission from reference [47].

carbonyl of PQQ, forming the hemiketal intermediate complex, which is further reduced to release the aldehyde product. This is illustrated in Figure 17. In the hydride transfer mechanism, after the proton abstraction by the base (Asp), the electrophilic C-5 carbonyl directly abstracts the methyl hydrogen as hydride releasing the aldehyde product.[46]

PQQ is also important for its antioxidant properties and as a growth promoting factor.[48] It has been reported that nanomolar oral consumptions of PQQ increases the B- and T-cells' responsiveness to mitogens and improves the reproductive outcomes and neurological function in rodents.[49] PQQ is post-translationally derived from Glu and Tyr amino acids. The process of its biogenesis has not been derived completely. Based on mutational studies, structural and functional analysis, and sequence homology, it is proposed that PQQ is derived from a precursor peptide PqqA, assisted by several *pqqA-F* gene products. The mechanism that is currently proposed for the cofactor biosynthesis is presented in detail elsewhere.[50]

Trihydroxyphenylalanine quinone (TPQ). Topaquinone is ubiquitously found in copper-containing amine oxidases from bacteria to humans, and participate in a wide variety of functions. Overall, the intrinsic cofactor facilitates the conversion of primary amine containing substrates to the aldehyde form, releasing ammonia in the process. The reaction, as for the other quinones, proceeds via a reductive cycle followed by the oxidative half of the reaction. Cofactor generation in amine oxidases occurs auto-catalytically and only requires molecular oxygen and the metal ion copper, which is coordinated by neighbouring histidines. Mutagenesis experiments of the active site Tyr 305 from the amine oxidase of *Hansenula polymorpha* have established that the oxido-reductive reaction requires this residue.[26a]

Backbone modifications.

Ammonia lyases. The degradation of aromatic amino acids follows a mechanism different from the mechanism generally followed for most of the other amino acids. Non-oxidative

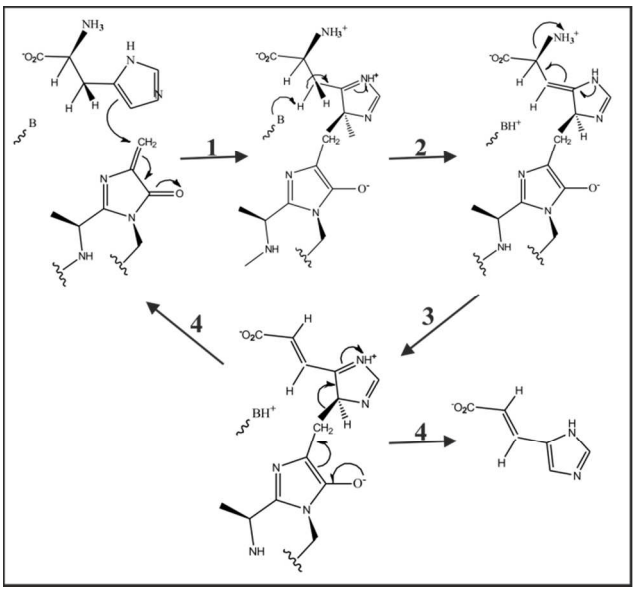

Figure 18. Mechanism of MIO action. Figure adapted with permission from reference [51]. Copyright (1999) American Chemical Society.

deamination of the aromatic amino acids histidine, phenylalanine and tyrosine, to ammonia, and their corresponding aryl acids having α, β unsaturated propeonic acid, is catalysed by enzyme histidine, phenylalanine, and tyrosine ammonia lyase, respectively.[51-52] It is believed that histidine ammonia lyase gave rise, evolutionarily through evolution of the metabolic pathway, to phenylalanine and tyrosine ammonia lyases.[52b, 52c]

Being the oldest enzyme, histidine ammonia lyase (histidase) is one of the most characterized of the three ammonia lyases. In the first step of histidine degradation, catalysed by histidase, the α-amino group is eliminated, resulting in the formation of an α-β unsaturated trans-urocanate. For this deamination reaction to be carried out, an electrophile is required. X-ray crystallographic studies show that the prosthetic group is an electrophile, 4-methylidene-imidazole-5-one (MIO).[51-52] This cofactor, formed by the spontaneous cyclization and dehydration of a tripeptide Ala-Ser-Tyr, is present in all three aromatic amino acid ammonia lyases.[51-52] The mechanism of formation of MIO is similar to green fluorescent protein (GFP; discussed later).[52a]

The mechanism by which the degradation is catalysed by these enzymes is shown in Figure 18. The process involves the electrophilic attack of histidine side chain on MIO (step 1 in Figure 18). This is followed by the C^{β} activation and proton removal by an enzymatic base (step 2). The removal of ammonia in step 3 of the catalytic process now facilitates the regeneration of MIO (and the enzyme active site) along with the release of the deaminated product (step 4).

The challenging catalytic elimination reaction of abstracting the C^{β} hydrogen necessitates that the neighbouring highly acidic amino group be maintained in a protonated form, so that it forms a good leaving group. This process requires an electrophilic group to catalyse the reaction. It has been shown by X-ray crystallographic studies, mutational studies and chemical modification experiments, that the MIO formed auto-catalytically from the Ala-Ser-Tyr triad acts as an excellent electrophilic group; the enhanced electrophilic nature as a result of the cyclization prevents delocalization of the nitrogen lone pairs of Ser and Tyr into the α, β unsaturated carbonyl system, thereby makes the non-oxidative deamination reaction of His, Tyr and Phe feasible.[51-52]

The catalytic triad formed by backbone cyclization was believed to be dehydroalanine for several decades, till electron cloud density in the X-ray crystallographic map of this protein (2.1 Å resolution) established the structure of MIO (Figure 19).[51] The presence of this modification makes histidase independent of cofactor (PLP, vitamin B12 and AMP), otherwise observed in other organisms.[51] Histidase catalyses the first step in degradation of histidine to urocanic acid, which undergoes further metabolism to generate glutamate. Malfunctioning of histidases results in a condition called histidenemia.

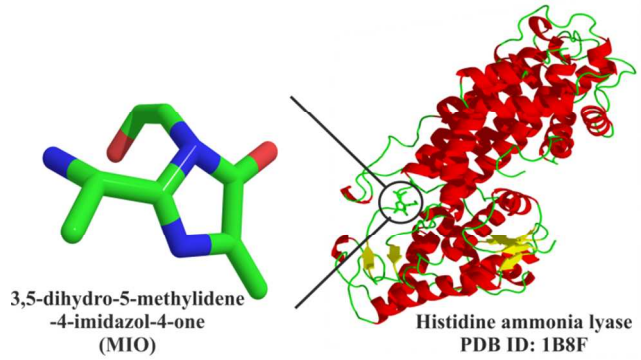

Figure 19. High resolution crystal structure of histidase, shown here in ribbon form, with the MIO cofactor highlighted.[51]

In tyrosine ammonia lyase from yeast *Rhodosporidium toruloides*, the MIO cofactor is formed by Ala 149, Ser 150 and Gly 151.[52c] Further, it is speculated that the MIO cofactor carries a covalently attached nucleophilic ammonium adduct. Two spatially proximal Tyr, a Gly and Arg residues are conserved in all the ammonia lyases, and provide anchoring interaction with the incoming substrate. Not surprisingly, mutation of these residues dramatically lowers the enzymatic activity of MIOs.[52c]

Similarly, phenylalanine ammonia lyase resembles histidase, with the exception of an ~200-residue N-terminal extension in the former, which controls active site access of the substrate.[52b] In plants, the MIO cofactor of this enzyme is generated by the Ala-Ser-Gly triad autocatalytically, by water elimination.[52b] It is proposed that this autocatalytic event is driven by mechanical pressure during the refolding process, similar to histidase. Further, the autocatalytic nature of prosthetic group generation

in this enzyme (and other lyases), ensures a cofactor-independent upregulation in parsley (*Petroselinum crispum*).[52b] Despite conservation and similarity between the ammonia lyases at the active site, loops positioned at the aromatic cluster of the active site discriminate between the three aromatic substrates and lowers cross-reactivity. For instance, it has been shown that in tyrosine ammonia lyase, a His 89 (numbering from *R. toruloides*) imidazole is involved in distinguishing Tyr from Phe and His.[52c] These enzymes are actively investigated for their medical relevance in phenylketonuria and other genetic diseases associated with amino acid metabolism.

Decarboxylases. Vitamin B6 gives rise to an important cofactor for several enzymes, called pyridoxal phosphate. Proteins that employ this cofactor, generally categorized under pyridoxal phosphate (PLP) proteins, and is required in reactions involving transamination, decarboxylation, racemization, elimination or replacement. One of the PLP proteins is histidine decarboxylase. This decarboxylase converts L-histidine to histamine, accompanied by the release of carbon dioxide, and is important in amino acid metabolism.
Surprisingly, histidine decarboxylase isolated from *Lactobacillus* 30a was found to work independent of PLP. The 2.5 Å crystal structure of this protein revealed that an intrinsic cofactor was generated by the auto-catalytic non-hydrolytic serinolysis of Ser 82 of the pro-enzyme (which becomes Ser 1 of the α-chain of the active enzyme). The pyruvate, thus formed from serine, forms a Schiff base with the substrate and facilitates the decarboxylation reaction mediated by PLP, in a PLP-independent manner.[1] *S*-Adenosylmethionine decarboxylase (AdoMetDC), involved in polyamine biosynthesis in bacteria, plants and humans, also undergoes internal post-translational serinolysis, generating the pyrovoyl moiety for decarboxylation. This has been reviewed extensively elsewhere.[53]

Protein tyrosine phosphatase 1B (PTP 1B). Human protein tyrosine phosphatases are signal transduction enzymes involved in tyrosine dephosphorylation and belong to two broad categories: receptor-like PTPs (RPTPs) and soluble cytosolic PTPs. The protein is usually ~250 amino acids, and the catalytic domain is highly conserved, with a 11-residue PTP signature motif (I/V)HCXAGXXR(S/T)G. Furthermore, the pK_a of the cysteine thiol group in this motif is ~5.4, whereas the normal pK_a is in the range of 9.0-9.5. This is necessary for the PTP 1B reaction, which proceeds via the formation of a cysteinyl phosphate intermediate during the first half of the reaction (phosphate abstraction from Tyr) and subsequent hydrolysis of this intermediate in the second half of the reaction.[54]
The enzyme is regulated by cellular hydrogen peroxide levels; the latter is required for numerous signal transduction pathways, particularly those mediated by tyrosine kinases. H_2O_2 acts by transiently inhibiting PTPs by converting the Cys-SH to Cys-S-OH. Indeed different oxidation states of the catalytic cysteine residue (sulphenic, sulphinic and sulphonic

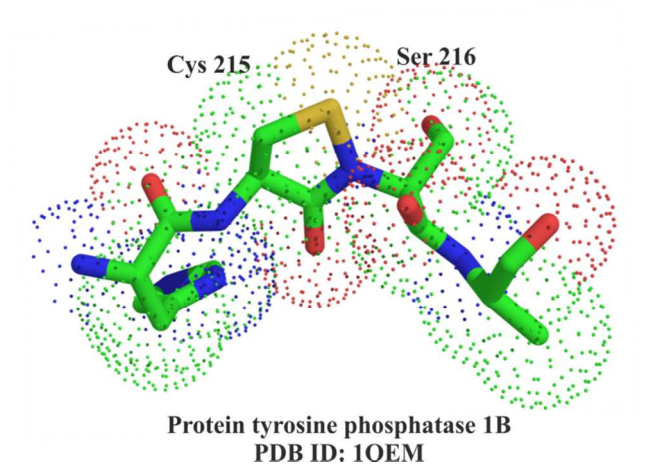

Figure 20. Five-membered puckered ring formed by a 1.7 Å sulphenyl-amide covalent bond formed between the S^{γ} of Cys 215 and backbone nitrogen of Ser 216 in protein tyrosine phosphatase 1B.[54]

acid derivatives) have been observed in the crystal structure.[54] Some of these oxidation states, formed in the presence of excess H_2O_2, can irreversibly inhibit the enzymatic activity.
How is irreversible inhibition prevented in the cell? The answer to this question came with the crystal structure of PTP 1B, with unusual electron density close to the side chain of the catalytic cysteine. A bond length of 1.7 Å, measured between S^{γ} of Cys 215 and the backbone nitrogen of Ser 216,[54a, 54b] could only be explained by the formation of a five-membered puckered ring harbouring a S-N covalent bond (Figure 20). It has been proposed that the sulphenyl-amide bond is generated by the nucleophilic attack of the amide on the sulphenic acid form of the cysteine side chain. The cysteine is regenerated by another nucleophilic attack on the S-N bond.[54a, 54b]
It has been proposed that the sulphenyl-amide intermediate is an elegant protection mechanism of the catalytic cysteine residue (pK_a ~ 5.4) from irreversible oxidation.[54a, 54b] The associated conformational changes due to the backbone constraint arising from the puckered five-member ring can signal an inactive state of the enzyme. Further, this sulphenyl-amide state of the protein can readily be restored, allowing for the re-activation of PTP 1B.

Cross-links in non-enzymatic proteins

Cross-linking between side chains, side chain and backbone and other backbone modifications, are also observed in a small, yet significant, class of non-enzymatic proteins. Some of these modifications, such as Tyr-Tyr cross-link in bovine γB crystallin forms due to ageing, whereas a similar Tyr-Tyr cross-link in extrusin from tomato cell wall confers rigidity to the structure. This section highlights some of the known and interesting examples identified in structural proteins, which may provide additional unique characteristics to the protein

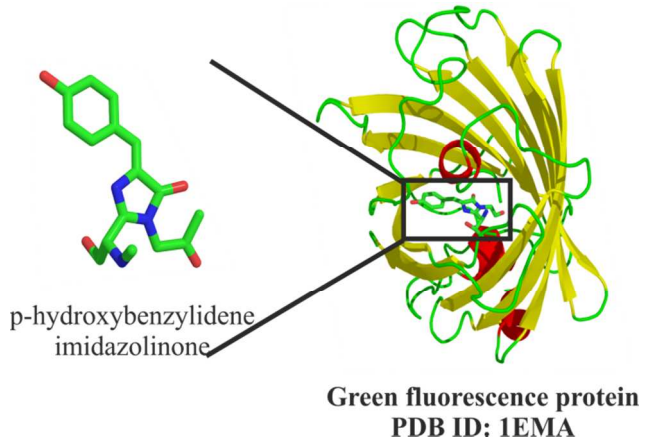

Figure 21. Ribbon diagram of GFP, highlighting the chromophore generated by backbone cyclization, in stick form.[55]

molecule. A small note on unique PTMs observed in peptides has also been included.

Green fluorescent protein (GFP). The ~27 kDa green fluorescent protein, known popularly as GFP, is one of the most prevalent and widely used protein for reporter-based gene expression, protein localization tags, biosensors, and several other applications in bacteria, yeast and higher organisms including mammalian systems, since its first application in ~1992 by Douglas Prasher.[56] This protein was first identified and isolated from the jellyfish *Aequorea victoria*,[57] and is an 11-stranded barrel with six short helical structures. Despite widespread applications of GFP, the term 4-(*p*-hydroxybenzylidene) imidazolidin-5-one (HBI) is seldom known in the GFP user community. The distinctive fluorescent nature of GFP is due to this unique chromophore HBI (Figure 21), formed at the centre of the barrel between residues Ser/Thr 65 – Tyr 66 – Gly 67 when GFP is correctly folded. Detailed mechanistic examination of this protein has been possible after the crystal structure was solved,[55] which has also serendipitously led to the development of several other fluorescent analogues of GFP by modulating the HBI chromophore within the barrel. Our current understanding of this protein as well as its derivatives is a result of extensive work carried out on GFP and its applications by several scientists including Osamu Shimomura, Martin Chalfie and Roger Y. Tsien, which fetched them the Nobel Prize in Chemistry in the year 2008.

Information on the application on GFP and its analogues is extensively described in several reports; the formation of the HBI chromophore itself is discussed here. The fluorescent nature of the chromophore arises due to an unusual covalent bond formed by backbone cyclization of residues Ser65 (backbone carboxyl group) and Gly 67 (backbone amide) followed by dehydration leading to the formation of the imidazolinone. The final oxidation reaction of the Tyr 66 C^{α}-C^{β} bond that uses molecular oxygen conjugates the phenolic ring with the imidazolinone. The phenolate ion thus generated serves as the light emitter. Presence of an unusual post-translational protein modification leading to chromophore formation was first identified by papain cleavage of GFP. Characterization of the hexapeptide F64-Q69, which was generated during this process, led to identification of this unusual cyclization. The peptide, in isolation, is non-fluorescent, and requires the local environment generated by the folded protein to exhibit green fluorescence.

In native GFP from *A. victoria*, the blue-green light is emitted at a maximum wavelength of ~508nm;[58] the emission maximum can be modified based on the chemical nature of the residues involved in the conjugation and chromophore formation.[59] This extensive conjugation not only confers the chromophoric property to the protein but also shows considerable thermal stability after formation. The chromophore formation is an autocatalytic event and occurs in the absence of cofactors;[55a] the only requirements for complete cyclization is a proper folded conformation of the protein as well as temperatures of 30°C or above.[2b] Additionally, isomerization across the *exo*-methylene bond is inhibited by its geometry; this results in a highly efficient green light emission by minimizing non-radiative pathways for decay of the excited fluorophore. The mechanism of the biogenesis of the chromophore is described by the Tsien group.[2a]

Ranasmurfin. The formation of stable chromophores using unusual post-translational modifications is not restricted to GFP. A blue-green protein identified in the foam nests of the Malaysian tree frog is one of several proteins and macromolecules that confer stability and adhesion properties to the nest. This blue-green protein, identified as Ranasmurfin, is a 13 kDa homodimer, with each subunit containing three disulphides and a covalent lysyl tyrosyl quinone linkage (LTQ) established between Tyr 2 and Lys 31 as well as Lys 30 and Tyr 108 (Figure 22) in each subunit.[60] The Tyr 30 – Tyr 108 LTQ is additionally modified by a nitrogen atom to link the two subunits and give rise to a *bis*-LTQ linkage (a four-residue Lys-Tyr-N-Tyr-Lys link), which now covalently links the dimer. This *bis*-LTQ, along with two histidines, form a coordinate bond with zinc.

While the LTQ modification is observed in other proteins (discussed later), the extended aromatic system, along with the metal coordination, gives rise to π-cloud re-distribution across the amide-aromatic bonds, and thereby confers the characteristic blue chromophore to ranasmurfin, with an absorbance range of 500 – 700 nm and emission maxima at 680 – 700 nm. The chromophore has two ionisable groups with a pK_a of 6.0 and 9.0, which make it exhibit reversible spectral shifts with change in pH. The blue colour is also unaffected by reducing agents such as $NaBH_4$; however, the colour slowly fades on treatment with EDTA and DTT, and rapid bleaching occurs in the presence of NBS.[60b] While the mechanism by which the LTQ post translational modification occurs and its biological importance is unknown, the cross-link confers high

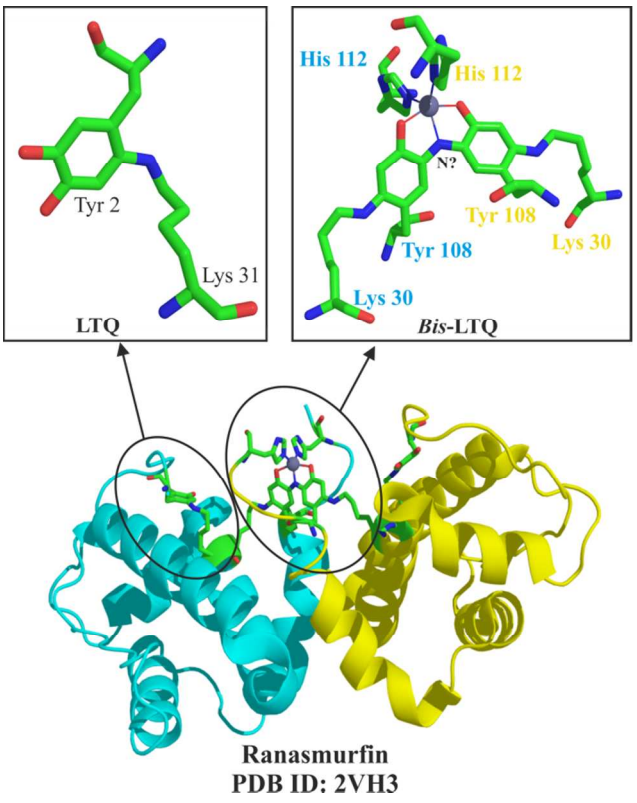

Figure 22. The unusual blue protein found in the Malaysian frog nest, Ranasmurfin, contains two subunits, each of which bears the unusual modification, LTQ, between Lys 31 and Tyr 108 (top, left). The *bis*-LTQ linkage is connected by an atom, speculated to be nitrogen (top, right). The ribbon diagram of ranasmurfin is provided in the bottom.[60b]

inter-subunit stability to the protein, resistance to partial proteolysis and denaturants, and it is speculated that the *bis*-LTQ plays a role in the mechanical adhesion properties of the foam.[60b]

Collagen. The basic components of basement membranes that underlie epithelia of all metazoans are collagen IV networks. They are formed from a family of six polypeptides chains (α1-α6). These associate into three different subtypes of protomers (α1α1α2, α3α4α5, and α5α5α6), which are triple-helical in nature. Self-assembly of the protomer occurs through end-to-end associations, wherein four protomers associate tail-to-tail through their amino termini. The carboxyl termini of two protomers, on the other hand, associate head-to-head through the noncollagenous (NC1) domains and give rise to dimers. The trimeric non-collagenous NC1 domains therefore exist as hexamers at the interface of the head-to-head interactions (Figure 23).[61]

NC1 domains of three monomer chains interact to form a trimer, which nucleates the formation of a triple helix. NC1 trimers from two protomers in the network assembly interact to form hexameric structure. Exposure to acidic pH or denaturants drives the dissociation of the hexameric assembly, yielding monomers and dimers. Existence of dimers in the denaturing conditions is because of the presence of cross-link(s) at the trimer-trimer interface.[62] These cross-links are present between the α1-like monomers: α1–α1, α1–α5, and α3–α5 and α2-like monomers: α2–α2, α2–α6, and α4–α4.[61-62]

It has been shown chemically, and by using X-ray crystallography (1.9 Å resolution), that the cross-link site of the trimer networks contains six unusual covalent thio-ether bonds between Met and Lys (Met 93 and Lys 211 in the case of human placenta collagen IV), formed through post-translational modifications (Figure 23).[63] There are two post-translational events believed to be involved in this novel bond formation. First, Lys is hydroxylated to Hyl (hydroxyl lysine) within the non-collagenous domain, which is followed by formation of the link between Hyl 211 and Met 93 of two trimeric non-collagenous domains.[61] The exact mechanism by which these post translational modifications occur is still unknown.

Surprisingly, X-ray crystallographic structure solved to a resolution of 1.5 Å did not show any supporting evidence for the existence of this cross-link (Figure 23).[64] On the contrary,

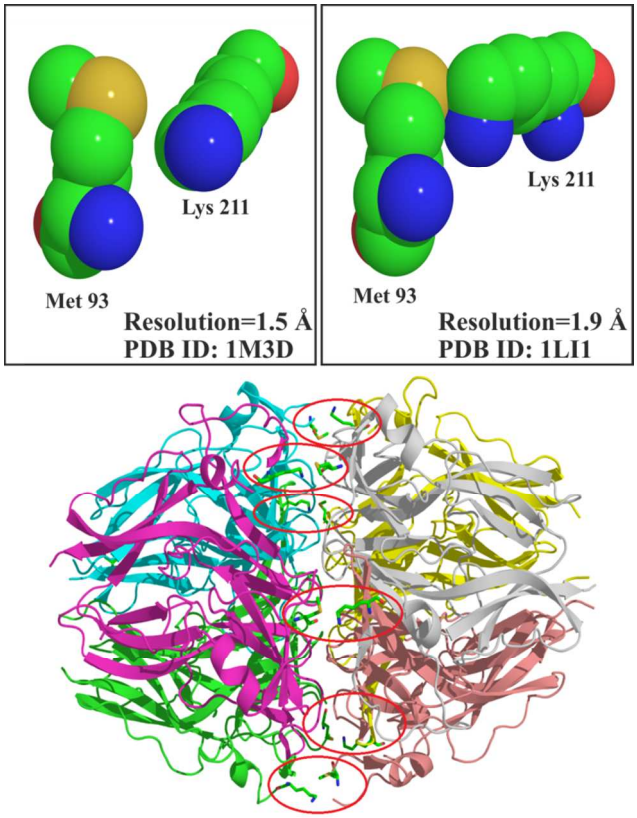

Figure 23. (Top) Crystal structures of collagen IV, highlighting the absence (left)[64-65] and presence (right)[63] of the cross-link at the oligomerization interface. Lys 211 – Met 93 interaction is represented here as spheres. The PDB IDs and resolution are indicated alongside the respective structures. (Bottom) The six unusual cross-links identified in the 1.9 Å crystal structure are highlighted. The protein is shown as ribbon diagram and residues involved in cross-linking are shown as sticks.[63]

mass spectrometric analysis, Edman degradation and amino acid analysis that were carried out using tryptic digests of the monomer and dimer subunits, revealed the existence of a novel cross-link between Hyl 211 and Met 93.[61] However, it is acknowledged that these covalent linkages confer the necessary structural stability for oligomerization, since monomers of the non-collagenous domains, which lack the cross-link are involved in Goodpasture disease and Alport syndrome.[63, 66]

Hemoglobin O. Truncated hemoglobins (trHb) are hemoproteins observed in microorganisms and are believed to be involved in cell respiration, particularly at the stationary phase of cell growth. The crystal structure of trHbO (a dodecamer, encoded by the *glbO* gene in *Mycobacterium tuberculosis* revealed an unusual cross-link between Tyr 36 $C^{\varepsilon 2}$ and a neighbouring Tyr 23 O (1.43 Å distance), which gives rise to rigidity between the two orthogonal aromatic rings. The cross-link positions the oxygen of Tyr 36 in the necessary geometry for stabilizing the heme-bound cyanide.[67] The ether link is speculated to occur in solution before dodecamer assembly through an unknown oxidative mechanism; its functional significance, besides establishing the necessary Tyr geometry, is still unclear.[67]

Peptides and toxins. Several examples of ribosomally and non-ribosomally synthesized peptides are known to bear unusual post-translational modifications. The unique protease resistance and structural stability conferred by these cross-links are critical for the function of these peptides. For example, bacteriocins, the antimicrobial bacterial peptides are cationic, ribosomally synthesized, are of 25 – 60 residues in length, and several of these molecules are extensively modified. For instance, nisin A from *Lactococcus lactis*, a food preservative and broad-spectrum bacteriocin, is 34 amino acids long and contains three unusual amino acids and five thio-ether bridges.[68] Similarly, subtilosin A, a *Bacillus subtimis* antimicrobial peptide, bears three S-C links between Cys and Phe/Thr, in addition to backbone cyclization.[69] Phalloidin, produced by the fungus *Amanita phalloides* is another bicyclic heptapeptide with a transannular thio-ether bridge connecting cysteine and tryptophan side chains.[70] The biosynthesis and unusual chemical properties of the ~ 20 distinct classes of PTM peptides is described in a recent review by the van der Donk team.[2i]

Conclusions

Crystallographic and biochemical studies have unearthed novel post-translational modifications within enzyme active sites that have structural and functional diversity. These modifications mainly serve as cofactors making these enzymes independent of usual cofactors found in proteins. Most of these enzymes bearing modifications carry out redox reactions, and usually involve a metal-dependent redox reaction in the establishment of the cross-link. The unusual post-translational protein modifications described in this review have remained alien for long, despite their evolutionarily conservation, with only few studies that report the chemistry behind their synthesis and their mode of action at the molecular level.

The advent of biophysical techniques has helped unravel some of these modifications, many of which continue to remain serendipitous discoveries. In the recent review by Klinman on quinone cofactors, she has commented on the evolutionary redundancy in enzymes that catalyse similar reactions.[26b] Furthermore, it is evident from our categorization of the identified unusual PTMs in this review, that a vast majority of these modifications are predominant in oxido-reductive (redox) reactions and in structural proteins. Cofactors for redox cycles are evolutionarily chosen for their ability to act as both electron donors and acceptors in different stages of the reaction, due to their extensively conjugated π-electron network. Unusual PTMs seem to cater to the requirement of the redox reactions, suggesting that more examples of such non-disulphide modifications can be obtained from such enzymes.

Redox reactions in proteins are oftentimes associated with the generation of free radicals; it is tempting to speculate that these unusual cross-links could have evolved due to accidental modifications at the protein active site by such radicals. Irrespective of their origin, one can safely assume that a larger collection of unusual PTMs will be unearthed in the oxidoreductase family of enzymes. Could these cross-links also perform alternate roles in protecting the enzyme active site from redox damage? As many cross-links show reversibility during catalysis in the protein, it would be of interest to examine their role in the regulation of protein activity under oxidation stress in the host cell, as well as their possible role in cell signalling.[54b]

Another major property of such PTMs is the associated rigidity they confer to proteins, especially at oligomerization interfaces. Hence, one other possible family of proteins that would possess such modifications would include the structural proteins. It would be possible to discover such PTMs using high resolution crystallography, coupled with mass spectrometric mapping, as employed earlier for PTP 1B,[54b] if such proteins are actively explored for unusual modifications. Surprisingly, however, in the case of GFP, ranasmurfin and other unusual chromophores, it has been speculated that these molecules evolved from related autocatalytic mechanisms by simple residue substitutions at the modification site to achieve the diverse chromophores.[71] Previous studies have also speculated that such modifications were retained by natural selection, due to their role in alternate, hitherto unknown, functions.[71] It would be of interest to identify such functions and develop synthetic mimetics that have regulatory roles in key biochemical processes.

The range of methodologies currently available for *in vitro* synthesis of such unusual cross-links does not span all the combinations generated in nature between the various amino acids. Nevertheless, the few strategies that have indeed been successfully developed to obtain di-tryptophan and di-tyrosine cross-links[72] in small peptides and proteins not only have potential applications in the generation of artificial enzymes,

but also serve to pave the way for more complex chemistries for production of many other unusual modifications.

Deducing the mechanism of formation of such unusual cross-links between amino acid side chains *in vivo* would facilitate the development of reagents that would allow their generation in *in vitro* systems. The scientific community has now acknowledged that non-disulphide cross-links are also possible in proteins and the observation of unusual or anomalous electron density in protein crystal structures, for example, need not be artefacts of data collection. Such cross-links, in addition to being of extreme interest themselves, can be exploited in the design and chemical synthesis of small peptide molecules that can be engineered to carry out protein-like reactions. Such catalytic peptides are of great scope in both industries and in curing diseases caused by enzyme deficiencies.

There is an escalating interest in the application of these cross-links to the generation of artificial enzymes and synthetic scaffolds with cofactor-independent catalytic activity. By mimicking the algorithms followed by nature in bringing about these modifications, it is possible to design strategies in drug targeting, create modified enzymes with a minimal effect on the geometry of the active site, which can perform catalytic activity devoid of cofactors and also to provide extra structural stability to the proteins. It would therefore come as no surprise that many more of such examples are uncovered or generated over the years, in a wider repertoire of proteins and peptides that will eventually give rise to a crunching number of such unusual post-translational modifications, and will no longer leave them unusual.

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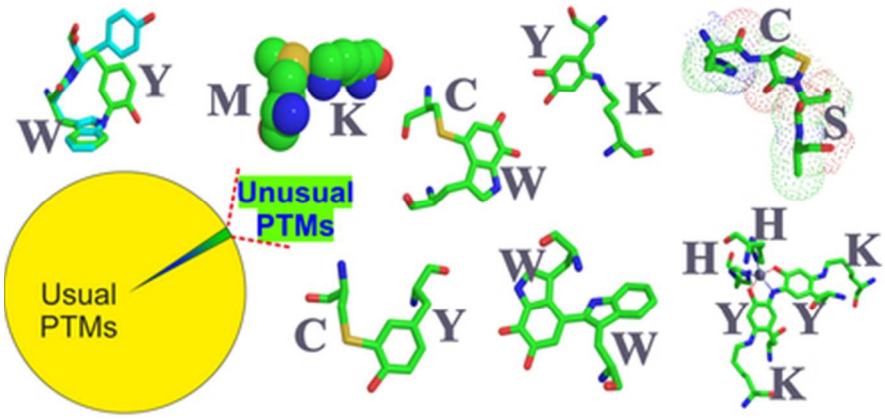

This review summarizes the "seemingly bizarre", yet naturally occurring, covalent non-disulphide keystones in enzymatic and scaffolding proteins, and their functions.
38x18mm (300 x 300 DPI)