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Current Topics

Protein-Derived Cofactors. Expanding the Scope of Post-Translational Modifications†

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ABSTRACT: Recent advances in enzymology, structural biology, and protein chemistry have extended the scope of the field of cofactor-dependent enzyme catalysis. It has been documented that catalytic and redox-active prosthetic groups may be derived from post-translational modification of amino acid residues of proteins. These protein-derived cofactors typically arise from the oxygenation of aromatic residues, covalent cross-linking of amino acid residues, or cyclization or cleavage of internal amino acid residues. In some cases, the post-translation modification is a self-processing event, whereas in others, another processing enzyme is required. The characterization of protein-derived cofactors and their mechanisms of biogenesis introduce a new dimension to our current views about protein evolution and protein structure−function relationships.

Amino acid residues at the active sites of enzymes play critical roles in catalysis. However, of the 20 commonly occurring amino acids in proteins, relatively few are chemically well suited to function as catalysts. Most amino acid side chains are essentially inert. Those that are most commonly seen functioning in enzyme active sites, such as histidine, serine, cysteine, and carboxylic residues, are limited in function to acid−base chemistry and electron donation during nucleophilic catalysis. To circumvent the limited chemical versatility of amino acids many enzymes utilize cofactors. A cofactor is a metal (e.g., iron or copper), an organic compound (e.g., pyridoxal phosphate or flavin), or an organometallic compound (e.g., heme or cobalamin) that is required for enzyme activity. The enzymes are initially synthesized as apoenzymes lacking activity until they combine with the cofactor to form the active holoenzyme. A tightly bound metal or organic compound required for enzyme activity is sometimes referred to as a prosthetic group. It has recently been demonstrated that certain enzymes have evolved an alternative method to circumvent the need for such exogenous cofactors. They utilize protein-derived cofactors, which are catalytic or redox-active centers that are formed by post-translational modification of one or more amino acid residues. The biosynthesis of these protein-derived cofactors typically involves the oxygenation of aromatic residues, covalent cross-linking of amino acid residues, or cyclization or cleavage of internal amino acid residues. The resulting cofactors often function at the enzyme active site either by providing an electrophilic site to interact with substrates or by stabilizing free radical intermediates, functions that the unmodified amino acid residues are incapable of performing.

Protein-derived cofactors add a new dimension to our view of the field of post-translational modification of proteins. The amino acid sequence of a protein is determined from the sequence of the gene from which it is encoded. It has long

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been recognized that after the completion of translation on the ribosome many newly synthesized proteins require further post-translational modifications to be able to perform their biological function. Typical post-translational modifications include phosphorylation, methylation, glycosylation, prenylation, and proteolytic cleavage to remove a signal or localization presequence. These well-characterized post-translational modifications primarily serve to regulate the biological activity of the protein, usually an enzyme, or to target the protein to its site of action in the cell. However, in contrast to the modifications that generate protein-derived cofactors, the modified amino acid residues do not typically acquire any new functional properties as a consequence of these post-translational modifications.

Protein-derived cofactors have for the most part gone unrecognized until recent years because they cannot be identified from the gene sequence for the protein. For most of the known protein-derived cofactors, there is no known sequence motif which is predictive of the site of modification. The presence of protein-derived cofactors has typically been established when high-resolution X-ray crystal structures of the host enzymes became available and advanced mass spectrometry technology allowed the identification of the modified peptides and amino acid residues. It is likely that additional protein-derived cofactors will be revealed as more structures of enzymes become available. The current status of this rapidly developing area of biochemical research is summarized in this work.

TYROSINE-DERIVED QUINONE COFACTORS

TPQ. The first protein-derived quinone cofactor to be characterized was topaquinone (2,4,5-trihydroxyphenylalanine quinone, TPQ[1], Figure 1), the prosthetic group of the copper-containing amine oxidases (*1*). This class of enzymes is found in bacteria, plants, and animals. These amine oxidases utilize a wide range of substrates and are involved in a wide range of physiological functions. The catalytic mechanism consists of two half-reactions (*2*). In the reductive half-reaction (eq 1), the amine substrate forms a covalent adduct with TPQ, which is reduced by two electrons and then releases the aldehyde product yielding an aminoquinol intermediate. In the oxidative half-reaction (eq 2), the aminoquinol is re-oxidized to TPQ by O_2, releasing NH_3 and H_2O_2.

$$E_{oxidized} + R\text{-}CH_2NH_2 \rightarrow E_{reduced} - NH_2 + R\text{-}CHO \quad (1)$$

$$E_{reduced} - NH_2 + O_2 + H_2O \rightarrow E_{oxidized} + NH_3 + H_2O_2 \quad (2)$$

The biogenesis of the TPQ cofactor, which requires the insertion of two oxygens into a specific tyrosine residue, has been shown to be a self-processing event (*3*, *4*). The recombinant enzyme has been expressed in copper-depleted *E. coli* cells and isolated as a copper-free apo-form in which the tyrosine residue has not been modified. Generation of

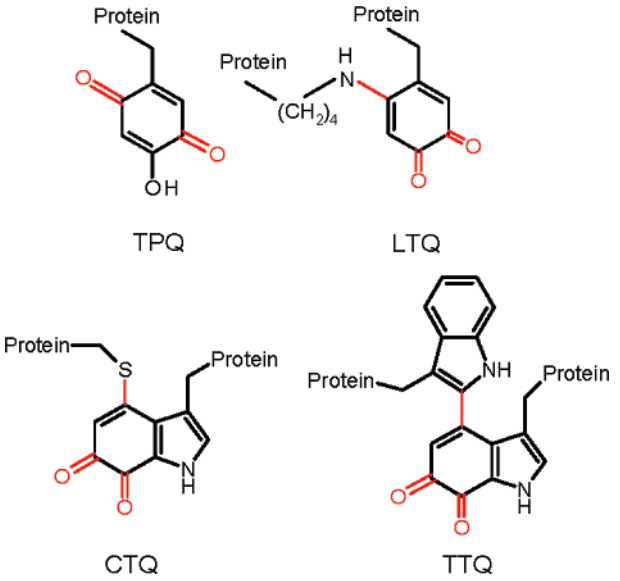


FIGURE 1: Protein-derived quinone cofactors. Post-translational modifications are highlighted in red. TPQ, 2,4,5-trihydroxyphenylalanine quinone; LTQ, lysine tyrosylquinone; CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone.

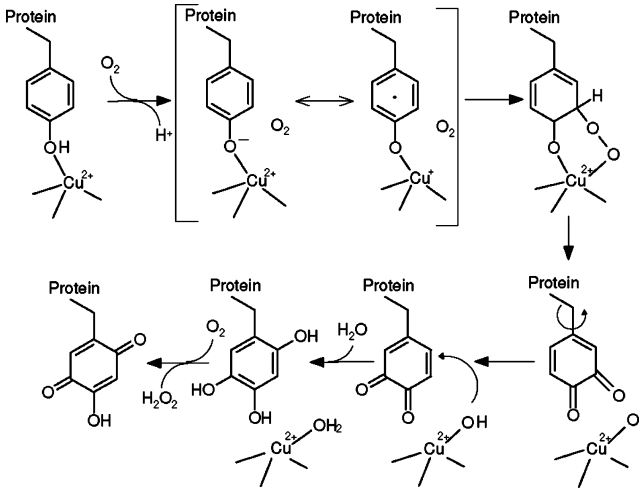


FIGURE 2: Proposed mechanism of TPQ biogenesis.

the active amine oxidase with correctly formed TPQ is achieved on addition of copper and exposure to oxygen. The tyrosine residue that is post-translationally modified is found within the highly conserved sequence of TXXNY(D/E) in which the conserved tyrosine residue is modified to form TPQ. This is a rare example of a predictive primary sequence motif for a protein-derived cofactor.

The mechanism proposed for TPQ biogenesis is shown in Figure 2 (*5*−*7*). Sites for copper and oxygen binding are in close proximity to the tyrosine that is modified. After deprotonation of the tyrosine, electron transfer from either tyrosinate-Cu^{2+} or Cu^+-tyrosyl radical to molecular oxygen yields superoxide, which then reacts with the tyrosyl radical to generate a copper-coordinated peroxide intermediate. In considering this mechanism, it should be noted that Ni(II) can support TPQ synthesis in *Hansenula polymorpha* amine oxidase (*8*), and Ni(II) and Co(II) can support TPQ formation in *Arthrobacter globiformis* amine oxidase (*9*). Fragmentation of this intermediate results in the formation of an ortho-quinone and copper-coordinated hydroxide. The side chain of the modified tyrosine then rotates 180° around its C_β−C_γ

[1] Abbreviations: TPQ, 2,4,5-trihydroxyphenylalanine quinone; LTQ, lysine tyrosylquinone; CTQ, cysteine tryptophylquinone; TTQ, tryptophan tryptophylquinone; MADH, methylamine dehydrogenase; AADH, aromatic amine dehydrogenase; QHNDH, quinohemoprotein amine dehydrogenase; MIO, methylidene-imidazoline; GFP, green fluorescent protein.

bond, after which addition of hydroxide at C2, followed by aromatization yields 2,4,5-trihydroxyphenylalanine. This is then oxidized by a second molecule of O_2 to form TPQ and H_2O_2. Evidence for the rotation of the modified tyrosine during cofactor biogenesis is supported by crystal structures of the apoprotein, and the zinc-substituted protein in which the post-translational modification has not occurred (*7*, *10*).

It has been demonstrated by site-directed mutagenesis that a second strictly conserved tyrosine residue that is present in the active site is also required for TPQ biogenesis. Different mutations of Tyr305 in *Hansenula polymorpha* amine oxidase selectively affected catalysis and TPQ biogenesis (*11*). During catalysis, it is proposed that Tyr305 is positioned to serve as a proton shuttle to O_2 during the oxidative half-reaction and may also play a role in maintaining TPQ in its reactive conformation. During TPQ biogenesis, Tyr305 could either function as an active-site acid for the breakdown of a proposed aryl-peroxy-Cu^{2+} intermediate or as an active-site base facilitating the removal of the C3 proton during the breakdown of that intermediate (*11*). Tyr305 could also serve a structural role, either in facilitating or restricting rotations and movement of intermediates during TPQ maturation (*7*). These results indicate that in addition to the modified tyrosine, at least one other active-site residue has multiple roles during cofactor biogenesis and catalysis. For the amine oxidases, it appears that in addition to the modified tyrosine, both copper and another active site tyrosine residue play roles in the biosynthesis of the protein-derived TPQ cofactor and then participate in the catalytic mechanism of the mature enzyme.

LTQ. Lysine tyrosylquinone (LTQ, Figure 1) is the protein-derived cofactor of mammalian lysyl oxidase, an important enzyme in the metabolism of connective tissue. Lysyl oxidase catalyzes the post-translational modification of elastin and collagen (*12*). It oxidizes selected peptidyl lysine residues to peptidyl α-aminoadipic δ-semialdehyde residues, which initiate formation of the covalent cross-linkages that insolubilize these extracellular proteins. This enzyme also contains copper as a second prosthetic group. The LTQ cofactor is formed by post-translational modification in which one atom of oxygen is incorporated into the tyrosine ring, and a covalent bond is formed between the tyrosine ring and the side-chain nitrogen of a lysine residue (*13*). This bond is formed at the same position at which the last oxygen atom is inserted into the mature TPQ cofactor in the amine oxidases. The mechanism of LTQ biogenesis has not yet been characterized. LTQ enzymes do not exhibit the conserved sequence motif that is characteristic of TPQ enzymes. Given the presence of copper at the active site of TPQ enzymes, it seems reasonable to assume that the mechanism of biosynthesis of LTQ is similar to that described earlier for TPQ (Figure 2) but with the lysine nitrogen adding to the orthoquinone intermediate rather than hydroxide.

TRYPTOPHAN-DERIVED QUINONE COFACTORS

TTQ. Tryptophan tryptophylquinone (2′,4-bitryptophan-6,7-dione, TTQ, Figure 1) (*14*) is the protein-derived cofactor of bacterial methylamine dehydrogenase (MADH) and aromatic amine dehydrogenase (AADH) (*15*). These are soluble enzymes localized in the periplasmic space of gram negative bacteria. Each is an inducible enzyme that allows the host bacterium to utilize particular primary amines as a sole source of carbon and energy. The catalytic mechanism consists of reductive and oxidative reactions (*16*). In the reductive reaction, the amine substrate forms a covalent adduct with TTQ that is reduced by two electrons and then releases the aldehyde product yielding an aminoquinol intermediate (*17*) (eq 3). The oxidative reactions involve interprotein electron transfer, usually to a type 1 copper protein. For most MADHs the electron acceptor is amicyanin (*18*), and for AADH, it is azurin (*19*). Because these copper proteins are one-electron carriers, the oxidation of the aminoquinol to TTQ requires two one-electron transfers. The product of the first electron-transfer is an aminosemiquinone (*20*) (eq 4). The immediate product of the second electron-transfer is an aminoquinone, which is hydrolyzed to release the ammonia product and regenerate the oxidized TTQ (*21*) (eq 5).

$$E_{oxidized} + CH_3NH_2 \rightarrow E_{reduced} - NH_2 + HCHO \quad (3)$$

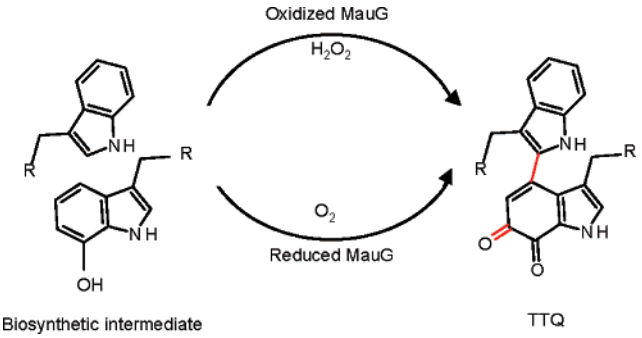
$$E_{reduced} - NH_2 + Amicyanin(Cu^{+2}) \rightarrow E_{semiquinone} - NH_2 + Amicyanin(Cu^{+1}) \quad (4)$$

$$E_{semiquinone} - NH_2 + Amicyanin(Cu^{+2}) \rightarrow E_{oxidized} + Amicyanin(Cu^{+1}) + NH_3 \quad (5)$$

TTQ is formed by a post-translational modification of two tryptophan residues of the polypeptide chain. Two atoms of oxygen are incorporated into the indole ring of one of the tryptophan residues, and a covalent bond between the indole rings of the two tryptophan residues is formed. The mechanism by which this occurs has not been completely elucidated, but it is known to require the action of other enzymes that are subject to the same genetic regulation as the structural genes for the enzyme (*22*, *23*). In *Paracoccus denitrificans*, the genes that encode the MADH subunits, together with nine other genes that relate to MADH expression and function, are clustered in the methylamine utilization (*mau*) locus with a gene order of *mauRFBEDACJGMN* (*22*, *23*). The first gene, *mauR*, is a Lys R-type transcriptional activator (*24*). The structural genes for the α and *β* subunits of MADH are *mauB* and *mauA*, respectively, and *mauC* encodes amicyanin (*25*), which is the obligate electron acceptor of MADH (*18*). The *β* subunit contains the tryptophan residues that are modified to TTQ, with *β*Trp57 incorporating the two oxygens and being cross-linked to *β*Trp108 (*26*). On the basis of sequence comparison, the last two genes, *mauM* and *mauN*, appear to encode ferredoxin-like proteins with unknown function (*23*). Four other genes, *mauFEDG*, were each shown to be essential for MADH biosynthesis (*22*, *23*). It has been shown that the gene product of *mauG*, a di-heme enzyme MauG (*27*), is able to catalyze the insertion of the second oxygen and the covalent bond between indole rings during the biosynthesis of TTQ in MADH (*28*, *29*).

The biogenesis of TTQ during MADH biosynthesis was studied using a recombinant expression system that contains the structural genes for MADH as well as the four other genes that are required for MADH biosynthesis (*30*). To test the role of MauG in TTQ biogenesis, the *mauG* gene was either deleted or inactivated by site-directed mutagenesis,

Scheme 1



and wild-type MADH was expressed in the background of the missing or inactive MauG. The resultant MADH was characterized by mass spectrometry and electrophoretic and kinetic analyses (*29*). The majority species that was isolated was a biosynthetic intermediate of MADH with incompletely synthesized TTQ containing a mono-hydroxylated βTrp57 and no covalent cross-link to residue βTrp108. Incubation of this intermediate in vitro with purified MauG resulted in the completion of TTQ biosynthesis and the formation of active MADH. Various electron donors could provide reducing equivalents for MauG-dependent TTQ biosynthesis under aerobic conditions (*31*), and under anaerobic conditions in the absence of an electron donor, H_2O_2 could serve as the source of oxygen and electrons for MauG-dependent TTQ biosynthesis (Scheme 1).

During the reaction with H_2O_2, a discrete reaction intermediate was observed, which spectroscopically resembles the reduced quinol form of TTQ that then is oxidized to the quinone. These results suggest that not only the incorporation of oxygen into the mono-hydroxylated biosynthetic intermediate but also the subsequent oxidation of quinol MADH during TTQ biosynthesis may be MauG-dependent processes (*31*). It was also shown that TTQ formation is linked to the proper assembly of α and β subunits during MADH biogenesis (*28*, *29*). In the absence of the TTQ cross-link, the association of the protein subunits is weaker than that in the mature enzyme. In mature MADH, a loop comprising residues β90−β108, which occupies about 50% of the subunit−subunit interface, is anchored in place by the cross-link between βTrp57 and βTrp108. It was proposed that in the absence of the cross-link, this loop could open, allowing the transient dissociation of subunits and access to βTrp57 by a processing enzyme such as MauG (*29*).

The mechanism by which the first oxygen is inserted into βTrp57 is unknown. However, relatively conservative βD76N and βD32N mutations of two aspartate residues that are located in close proximity to the quinone oxygens of TTQ in *P. denitrificans* MADH resulted in very low levels of MADH expression (*32*). Analysis of the isolated proteins by mass spectrometry revealed that each mutation affected TTQ biogenesis (*33*). βD76N MADH was completely inactive, and the isolated β subunit possessed the six disulfides seen in the mature protein but had no oxygen incorporated into βTrp57 and no cross-link. The βD32N MADH preparation contained a major species with six disulfides but no oxygen incorporated into βTrp57 and a minor species with mature TTQ, which was active. Thus, while the mechanism remains unknown, it appears that these active-site aspartic acid residues are critical for insertion of the first oxygen into βTrp57 during TTQ biogenesis.

CTQ. Cysteine tryptophylquinone (CTQ, Figure 1) is the protein-derived cofactor of quinohemoprotein amine dehydrogenase (QHNDH) (*34*). CTQ is formed by post-translational modifications during which two atoms of oxygen are incorporated into the indole ring of a tryptophan residue and a covalent bond is formed between the modified indole ring and the sulfur of a cysteine residue. The overall structures of the TTQ enzymes, MADH (*26*), and AADH (*35*) are similar. Each is a heterotetramer of two identical larger α subunits of molecular weight 40,000−50,000 and two identical smaller β subunits of molecular weight ~15,000, which contain TTQ. In contrast, the CTQ-bearing QHNDH is an $\alpha\beta\gamma$ heterotrimeric protein (*34*). The smallest 82-residue γ subunit contains CTQ. In addition to CTQ, the γ subunit contains three novel thioether cross-links that are formed between cysteine sulfurs and either the β- or γ-methylene carbon of an aspartic or glutamic acid residue. The α subunit is a four-domain polypeptide chain, which contains two *c*-type hemes. One heme *c* is solvent-accessible, and the other is fully buried within the α subunit and located approximately 9 Å from the tryptophylquinone moiety of CTQ on the γ subunit. The α and γ subunits sit on the surface of the β subunit that with the γ subunit forms the enzyme active site. The reaction mechanism of QHNDH is essentially the same as that of the TTQ enzymes (*36*). The major difference is that the immediate electron acceptor for the reduced CTQ is the heme, which is present on the α subunit, rather than an exogenous copper protein.

Relatively little is known about the mechanism of the biogenesis of CTQ. The gene cluster that encodes QHNDH (*34*) is completely different from that which encodes MADH. Present in the QHNDH operon is an open reading frame (ORF2) that encodes a putative radical SAM protein (*37*), which is not present in the MADH operon. Site-specific mutations in the putative [Fe-S] cluster or SAM-binding motifs in the ORF2 protein show that the ORF2 protein is necessary for the post-translational processing of the γ subunit, most likely participating in the formation of the intrapeptidyl thioether cross-links (*38*). No gene analogous to *mauG* is present in the gene cluster. Although perhaps coincidental, it is interesting that a gene encoding a di-heme protein is also present in the QHNDH gene cluster, but in this case, the gene product is not a free cytochrome but rather a subunit of QHNDH that associates closely with the CTQ-bearing subunit. It is noteworthy that despite the gross structural differences between the TTQ and CTQ enzymes, the two aspartate residues required for the insertion of the first oxygen during TTQ biogenesis in MADH (discussed earlier) are structurally conserved in QHNDH with respect to the CTQ cofactor (*36*). Thus, it appears that for TTQ and CTQ enzymes, an active-site aspartic acid is necessary both for cofactor biogenesis and catalysis after cofactor maturation.

CROSS-LINKED AMINO ACID RESIDUES IN HEME ENZYMES

Covalently cross-linked amino acids have been identified in the proximity of the catalytic heme of certain redox enzymes (Figure 3). Three crystal structures of catalase-

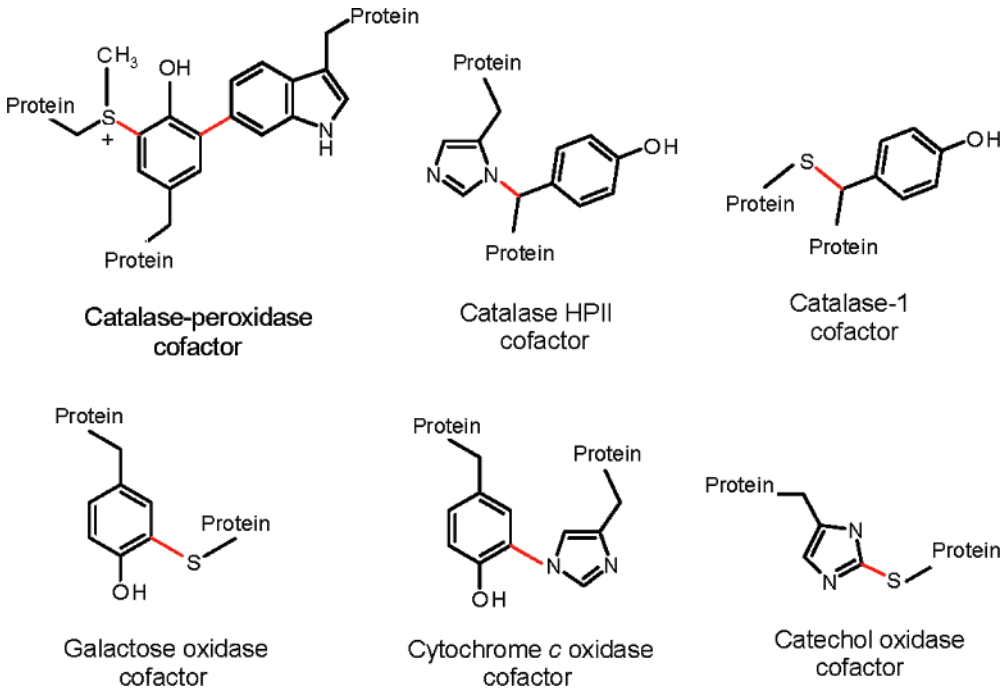


FIGURE 3: Cross-linked amino acids present in heme and copper proteins. Post-translational modifications are highlighted in red.

peroxidase (KatG) from different sources, *Haloarcula marismortui* (*39*), *Burkholderia pseudomonas* (*40*), and *Mycobacterium tuberculosis* (*40*), each reveal the presence of two covalent bonds between three amino acid side-chains, Tryptophan107, Tyrosine229, and Methionine255 (*M. tuberculosis* numbering), located on the distal side of the heme active site. This structural feature is not found in the monofunctional peroxidases, suggesting that these cross-linked amino acids may impart catalytic activity specifically to the KatGs. Mutagenesis studies have confirmed that the cross-link is required for catalatic but not for peroxidatic activity (*41*).

To gain insight into the mechanism of formation of the methionine−tyrosine−tryptophan cross-link, a protein lacking the cross-links was prepared by expressing the protein in cells deficient in heme by growth in iron-depleted media. After reconstitution with heme in vitro, the formation of the cross-links was observed after the addition of peroxyacetic acid (*42*). The following mechanism was proposed. The formation of compound I (oxo-ferryl porphyrin cation radical) on the heme leads to the oxidation of both Tyr229 and Trp107. Coupling of the two radicals occurs, resulting in the formation of the Tyr−Trp bond. The formation of a second compound I intermediate then results in the oxidation of the cross-linked Tyr−Trp leading to nucleophilic attack by the sulfur of Met255 forming the second cross-link. An interesting conclusion from these findings is that the active-site heme is required to generate the cross-linked amino acids during biosynthesis, and then the cross-linked amino acids are required by the heme to perform its catalytic function in the mature enzyme. Cross-linked amino acids have been observed in the active sites of two other heme enzymes. A histidine−tyrosine cross-link has been identified in catalase HPII (*43*), and a cysteine−tyrosine cross-link has been identified in catalase-1 (*44*) (Figure 3). In these enzymes, the bond is formed with the β-carbon of tyrosine rather than the aromatic ring as in KatG.

CROSS-LINKED AMINO ACID RESIDUES IN COPPER ENZYMES

Complex Copper Proteins. Cross-linked amino acids have also been identified at the active sites of certain copper proteins. The crystal structure of a plant catechol oxidase reveals that the Cu_A of the dinuclear Cu_A-Cu_B metal site is coordinated by three histidine ligands, one of which is covalently linked to a cysteine by a thioether bond (*45*) (Figure 3). A similar cysteine−histidine bond to one of the copper ligands in the dinuclear metal site of Octopus hemocyanin has also been identified in its crystal structure (*46*). The crystal structures of bacterial and mammalian cytochrome *c* oxidase (*47*, *48*), a key component of the membrane-bound respiratory chain, reveal that a covalent cross-link exists between the side chains of a tyrosine residue and a histidine residue (Figure 3), which provides one of the copper ligands of the binuclear to the Cu_B component of the Cu_B-$heme_{a3}$ center. This histidine−tyrosine cross-link is believed to be a common feature of each of the three distinct families of heme-copper oxygen reductases in nature (*49*).

Galactose Oxidase. One of the first published examples of the functional cross-linking of amino acid residues within a protein to generate a reactive prosthetic group is the protein-derived cofactor of the fungal enzyme galactose oxidase (Figure 3). The overall reaction catalyzed by galactose oxidase is the oxidation of a primary alcohol to the corresponding aldehyde, coupled to the reduction of dioxygen to hydrogen peroxide (*50*) (eq 6). The crystal structure (*51*) showed the presence of a thioether bond covalently linking Cysteine228 and Tyrosine272, with this tyrosine also acting as a ligand to the copper that is present in the active site.

$$RCH_2OH + O_2 \rightarrow RCHO + H_2O_2 \qquad (6)$$

The side chains of these two residues also form an aromatic plane that stacks with the indole ring of Trptophan290. Oxidation of Tyr272 generates a radical providing a metallo-

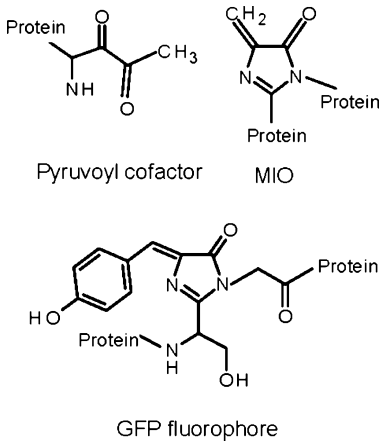


FIGURE 4: Protein-derived cofactors formed by cleavage or cyclization of internal amino acid residues. MIO, methylidene-imidazoline; GFP, green fluorescent protein.

radical complex of the tyrosyl radical coordinated to the copper in the active state of the enzyme (*50*). A similar tyrosine−cysteine cross-link has recently been seen in the active site of mammalian cysteine dioxygenase, a mononuclear iron-dependent enzyme (*52*).

Galactose oxidase is initially synthesized with a presequence that was proposed to be required for the generation of the galactose oxidase cofactor and is cleaved during processing to generate the mature enzyme. When cells expressing the enzyme are grown in copper-depleted media, the enzyme is produced in a precursor form lacking the thioether bond and also possessing an additional 17-amino acid presequence at the N terminus. The formation of the cofactor is a self-processing event. The tyrosine−cysteine cross-link forms spontaneously in vitro in the presence of O_2 and Cu^{2+}, yielding a free radical coupled-Cu^{2+} cofactor in the active site of the mature enzyme (*53*). The crystal structure of this precursor protein has been determined and compared to that of the mature protein (*54*). The presequence does not make direct contact with the active site, but it causes several structural elements to reside at positions different from those that they occupy in the processed protein. As a result, the tyrosine and cysteine residues to be cross-linked as well as the tryptophan that π-stacks with the cross-link after it is formed are in much different positions in the precursor than in the mature protein. The other residues that provide copper ligands are in similar orientations and positions in the precursor and mature protein, suggesting that the precursor binds copper at the site to initiate the post-translational modification events that not only generate the protein-derived cofactor but also alter the conformation of the protein to provide the functional active-site environment for catalysis. However, the role of the presequence was questioned in studies using a recombinant galactose oxidase precursor protein that is isolated with a processed N-terminus (i.e., no prequence) but no tyrosine−cysteine cross-link. Mature galactose oxidase could be readily formed in vitro from that precursor by addition of O_2 and Cu^{1+} (*55*).

OTHER PROTEIN-DERIVED CARBONYL COFACTORS

Pyruvoyl Cofactor. Pyruvoyl cofactor (Figure 4) (*56*) is derived from the post-translational modification of an internal serine residue, and it does not equilibrate with exogenous pyruvate. This class of enzymes plays an important role in the formation of biologically important amines and includes histidine and aspartate decarboxylases from bacteria, and *S*-adenosylmethionine decarboxylases, phosphatidylserine decarboxylases, and 4′-phosphate pantothenoylcysteine decarboxylases from bacterial and eukaryotic sources. The mechanism of action of pyruvoyl decarboxylases is believed to involve the formation of a Schiff base, a covalent adduct between the carbonyl carbon of the pyruvoyl cofactor and the α-amino group of the substrate. The decarboxylation reaction then proceeds via a mechanism similar to that catalyzed by pyridoxal phosphate dependent decarboxylases (*56*).

A common feature of this class of enzymes is that the catalytic pyruvoyl residue is covalently bound to the amino terminus of one of two nonidentical subunits. The two-subunit active enzyme is initially synthesized as an inactive single subunit proenzyme. Activation requires post-translational modification during which a specific internal serine residue becomes the pyruvoyl prosthetic group concomitant with the cleavage of the proenzyme. The chemical mechanism of pyruvoyl group formation is well-established (*56*) (Figure 5A). It occurs through nonhydrolytic serinolysis, in which the side-chain hydroxyl group supplies oxygen to form the C-terminus of the β chain, while the remainder of the serine residue is converted to ammonia and the pyruvoyl group that blocks the N terminus of the α chain. The serine oxygen attacks the carbonyl carbon to form a cyclic oxyoxazolidine intermediate, which converts to a linear ester intermediate (*57*). The ester undergoes β-elimination, creating dehydroalanine at the N-terminus of the site of cleavage and release of what becomes the β chain of the mature enzyme. Tautomerization of dehydroalanine produces an imine, which is converted first to a carbinolamine and then deaminated to form the covalently attached pyruvoyl group, which is present at the end of the α chain in the mature enzyme. Site-directed mutagenesis of this serine residue in the *S*-adenosylmethionine decarboxylase proenzyme showed that changing it to alanine completely prevented the processing and the formation of active enzyme (*58*) and allowed determination of the crystal structure of the unprocessed proenzyme (*59*).

Two other enzymes that contain the pyruvoyl cofactor are D-proline reductase and glycine reductase. These enzymes were originally reported to contain the pyruvate in an ester linkage, but later studies have demonstrated its presence at the amino terminus of one of the subunits by an amide bond. In contrast to the pyruvoyl-dependent decarboxylases, the site of internal cleavage and modification of these reductases is a cysteine rather than a serine (*60*, *61*).

MIO. Methylidene-imidazoline (MIO) (Figure 4) has been shown to be the prosthetic group of two enzymes, phenylalanine ammonia-lyase and histidine ammonia-lyase. The former is an important plant enzyme that eliminates ammonia from phenylalanine to form *trans*-cinnamic acid, a precursor of lignin, flavinoids, and coumarin. These enzymes catalyze the non-oxidative deamination of their respective substrates. Mechanistic studies of these enzymes suggest that MIO forms a covalent adduct with the side chain of its amino acid substrate during catalysis, which facilitates the elimination of ammonia to yield product (*62*).

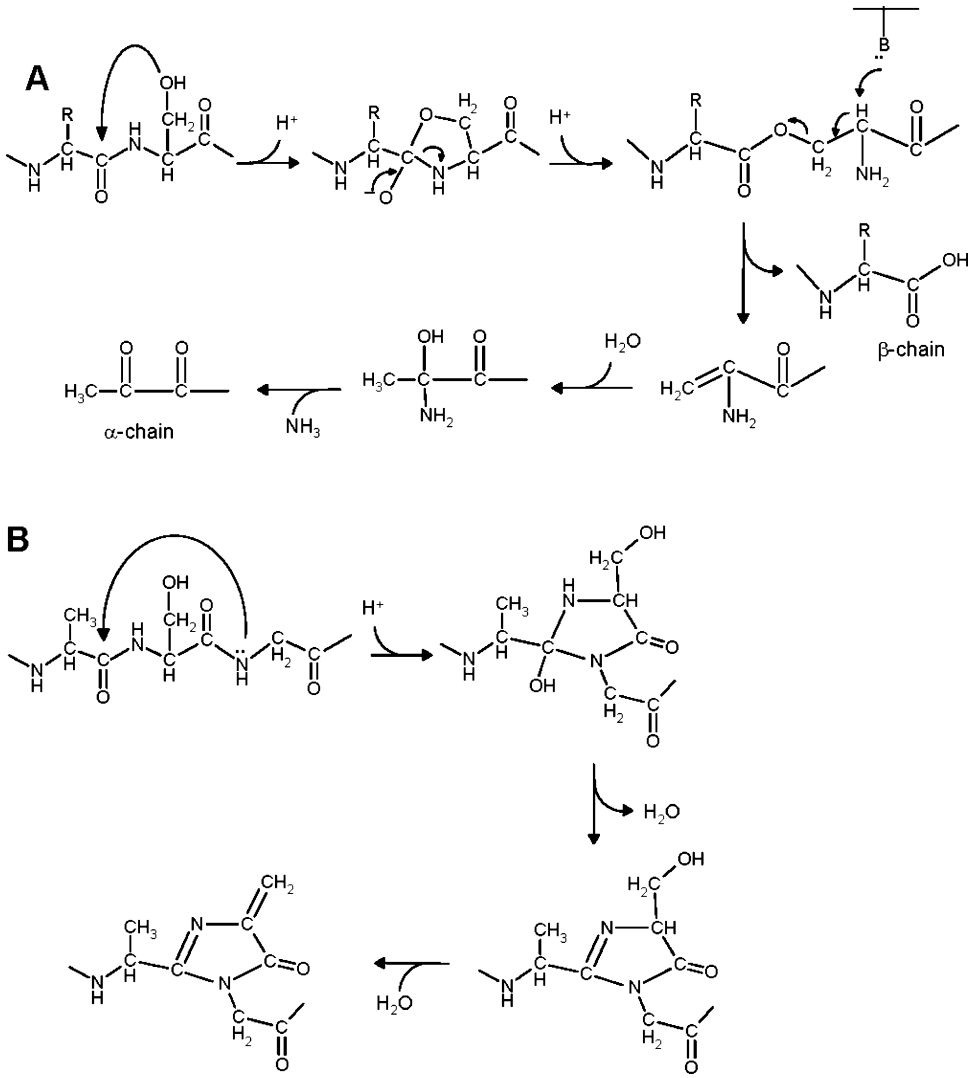


FIGURE 5: Proposed mechanisms of biogenesis of (A) pyruvoyl cofactor and (B) MIO.

This cofactor was previously thought to be dehydroalanine, but the crystal structure of histidine ammonia-lyase revealed that it was a modified dehydroalanine, MIO (*63*). On the basis of the results of site-directed mutagenesis and X-ray crystallographic studies, it was proposed that the mechanism of MIO biosynthesis in histidine ammonia-lyase occurs via self-processing reactions, cyclization of residues Alanine142 and Glycine144, followed by dehydration of Serine143 (Figure 5B).

GREEN FLUORESCENT PROTEIN CHROMOPHORE

The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*, although not an enzyme, has become widely used as a marker for gene expression and localization. It is discussed here because the X-ray crystal structure of this protein revealed that the covalently bound fluorescent chromophore is derived from three adjacent amino acids, serine−tyrosine−glycine on the polypeptide chain (*64*) (Figure 4). The formation of the chromophore involves self-processing reactions that bear similarity to the mechanism of the formation of MIO discussed earlier. It is proposed that formation occurs via condensation between the amide nitrogen of Gly67 and the carbonyl of Ser65, followed by autoxidation of the $\alpha-\beta$ bond of Tyr66 (*65*). It was subsequently shown that red and yellow fluorescent proteins could be formed naturally and via protein engineering as a result of the substitution of the serine residue with other amino acids (*66*). It should be noted that the isolated tripeptide will not undergo these reactions. For synthesis of the GFP cofactor to occur, the protein must be folded in such a manner to position the residues such that the biosynthetic reactions occur.

AMINO ACID-BASED FREE RADICALS

In addition to the protein-derived cofactors described above, it has been shown in many enzymes that specific amino acid residues may function in catalytic and electron-transfer reactions by stabilizing free-radical reaction intermediates. Examples of such amino acid radicals include tyrosyl radicals in ribonucleotide reductase, prostaglandin H synthase and photosystem II; tryptophyl radicals in DNA photolyase and cytochrome *c* peroxidase; a glycyl radical in pyruvate formate lyase; and a cysteine-based thiyl free radical in some ribonucleotide reductases. There is no evidence to date that these amino acid residues have been covalently modified. More information on this topic may be found in recent reviews (*67*−*71*).

CONCLUSIONS AND PERSPECTIVES

The identification of protein-derived cofactors and characterization of the mechanisms of their biogenesis require a re-evaluation of our current ideas about protein evolution and protein structure−function relationships. In each of the examples described here, the post-translational modification of the amino acid(s) to generate the protein-derived cofactor was dependent on the presence of other specific conserved amino acid residues or structural features of the protein, or both. In some cases, a proenzyme with a structure different from that of the mature enzyme is required for the biosynthesis of the protein-derived cofactor. In other cases, amino acid residues and bound metals have been shown to possess multiple functions in cofactor biogenesis as well as catalysis. Because cofactor biogenesis must come first, it follows that these residues and those binding the metal did not evolve for their current catalytic function. Rather, they evolved and were conserved for their role in cofactor biogenesis, or in some different catalytic function, that led to the generation of the protein-derived cofactor and a new function. The characterization of protein-derived cofactors also has implications for protein engineering. A better understanding of the mechanisms of the biogenesis of protein-derived cofactors will provide insight into how to design or modify the sequence of existing proteins so that they form protein-derived cofactors that introduce a catalytic or redox center within a protein without the need for the addition of exogenous cofactors.

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