

Prolyl 4-Hydroxylases and their Protein Disulfide Isomerase Subunit

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Abstract

Prolyl 4-hydroxylases (EC 1.14.11.2) catalyze the formation of 4-hydroxyproline in collagens and other proteins with collagen-like sequences. The vertebrate type I and type II enzymes are $[\alpha(I)]_2\beta_2$ and $[\alpha(II)]_2\beta_2$ tetramers, respectively, whereas the enzyme from the nematode *Caenorhabditis elegans* is an $\alpha\beta$ dimer. The type I enzyme is the major form in most but not all vertebrate tissues. The catalytic properties of the various enzyme forms are highly similar, but there are distinct, although small, differences in K_m values for various peptide substrates between the enzyme forms and major differences in K_i values for the competitive inhibitor, poly(L-proline). Prolyl 4-hydroxylase requires Fe^{2+} , 2-oxoglutarate, O_2 and ascorbate. Kinetic studies and theoretical considerations have led to elucidation of the reaction mechanism, and recent extensive site-directed mutagenesis studies have identified five critical residues at the cosubstrate binding sites. A number of compounds have been characterized that inhibit it competitively with respect to some of the cosubstrates, and three groups of suicide inactivators have also been identified. The β subunit in all forms of prolyl 4-hydroxylase is identical to protein disulfide isomerase (PDI), a multifunctional polypeptide that also serves as a subunit in the microsomal triglyceride transfer protein, as a chaperone-like polypeptide that probably assists folding of a number of newly synthesized proteins, and in several other functions. The main role of the PDI polypeptide as a protein subunit is probably related to its chaperone function. Recent expression studies of recombinant human prolyl 4-hydroxylase subunits in a yeast have indicated that the formation of a stable enzyme tetramer *in vivo* requires coexpression of collagen polypeptide chains.

Key words: chaperone, collagen, dioxygenase, prolyl 4-hydroxylase, protein disulfide isomerase.

Introduction

Prolyl 4-hydroxylase (EC 1.14.11.2) catalyzes the formation of 4-hydroxyproline in collagens and more than ten additional proteins with collagen-like sequences. This enzyme has a central role in the synthesis of all collagens, as the hydroxy groups of the 4-hydroxyproline

residues are essential for the formation of the collagen triple helix at body temperature. This critical function of 4-hydroxyproline makes prolyl 4-hydroxylase a potential target for therapeutic modulation of the excessive collagen accumulation found in patients with various fibrotic diseases and has prompted numerous studies on the enzyme (for more extensive reviews and detailed ref-

erences, see Kivirikko et al., 1992; Kivirikko and Pihlajaniemi, 1998).

The prolyl 4-hydroxylases from all vertebrate sources studied are $\alpha_2\beta_2$ tetramers. Although this over-all structure has been known for more than 25 years, all attempts to assemble an enzyme tetramer from its subunits *in vitro* have been unsuccessful. A fully active recombinant prolyl 4-hydroxylase tetramer has been produced, however, by coexpression of the two types of enzyme subunit in insect cells (Vuori et al., 1992b) and a yeast (Vuorela et al., 1997). This has made it possible to initiate extensive site-directed mutagenesis studies to identify functionally important amino acid residues of the enzyme and to perform detailed studies on its various properties. Coexpression of the enzyme subunits with collagen polypeptide chains has also made it possible to develop high-level expression systems for recombinant human collagens in insect cells, which have insufficient levels of endogenous prolyl 4-hydroxylase activity (Lamberg et al., 1996a; Myllyharju et al., 1997), and in yeast, which has no prolyl 4-hydroxylase activity at all (Vuorela et al., 1997).

The α Subunits Specify the Forms of Prolyl 4-Hydroxylase

Prolyl 4-hydroxylase had long been assumed to be of one type only, with no isoenzymes, but recently an isoform of the vertebrate α subunit, termed the α (II) subunit, has been cloned and characterized from man (Annunen et al., 1997) and from mouse (Helaakoski et al., 1995). Correspondingly, the previously known vertebrate α subunit, which has been cloned from human

(Helaakoski et al., 1989), rat (Hopkinson et al., 1994), mouse (Helaakoski et al., 1995) and chicken (Bassuk et al., 1989) sources, is now called the α (I) subunit. Both types form an $\alpha_2\beta_2$ tetramer with the same β subunit, the $[\alpha(I)]_2\beta_2$ and $[\alpha(II)]_2\beta_2$ tetramers (Fig. 1) being termed the type I and type II enzymes, respectively (Helaakoski et al., 1995). Data on coexpression in insect cells strongly argue against the presence of an $\alpha(I)\alpha(II)\beta_2$ tetramer containing both types of α subunit in a single molecule (Annunen et al., 1997).

The processed human α (I) and α (II) subunits consist of 517 and 514 amino acids, respectively, both being synthesized in a form containing a signal peptide of 17 or 21 additional residues (Helaakoski et al., 1989; Annunen et al., 1997). The over-all amino acid sequence identity between the processed human α (I) and α (II) subunits is 64%. This identity is not distributed equally, being highest within the C-terminal region, which contains many residues involved in cosubstrate binding sites (Fig. 2). Both types of α subunit contain five cysteine residues in identical positions, but the α (II) subunit contains an additional cysteine located between the fourth and fifth cysteines of the α (I) subunit (Fig. 2). Site-directed mutagenesis studies suggest that the second and third cysteines in the α (I) subunit form one intrachain disulfide bond and the fourth and fifth cysteines another, both these disulfide bonds being essential for the α subunits to maintain the native structures needed for tetramer assembly (John and Bulleid, 1994; Lamberg et al., 1995). The tetramers have no interchain disulfide bonds.

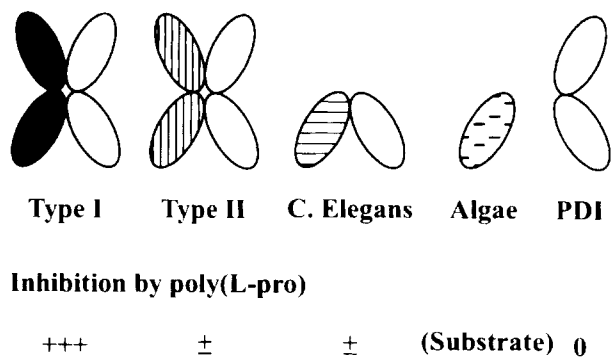


Figure 1. Schematic representation of the different forms of prolyl 4-hydroxylase. The PDI dimer and inhibition of the various prolyl 4-hydroxylase forms and PDI by poly(L-proline) are also shown.

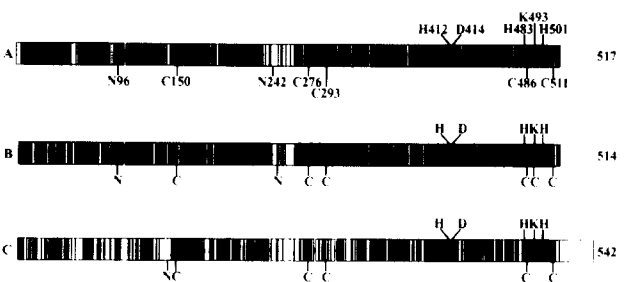


Figure 2. Schematic representation of the α (I) (A) and α (II) (B) subunits of human prolyl 4-hydroxylase and the α subunit of the *C. elegans* enzyme (C). Numbering of the amino acids begins with the first residue in the processed α subunits. Amino acids that are identical or similar between at least two of the α subunits are shown in black or grey, respectively. Cysteine residues, potential attachment sites for asparagine-linked oligosaccharides and the critical histidine, aspartate and lysine residues present at the cosubstrate binding sites are also shown.

The α (I) and α (II) subunits each contain two potential attachment sites for asparagine-linked oligosaccharides (Fig. 2). Site-directed mutagenesis studies on the human α (I) subunit have indicated that mutation of both these asparagines to glutamines causes no changes in tetramer assembly or in the catalytic properties of the tetramer (Lamberg et al., 1995). The extent of glycosylation of these two asparagines is variable, thus causing heterogeneity in the forms of the enzyme tetramer. An additional heterogeneity in forms of the type I enzyme is caused by mutually exclusive alternative splicing of RNA sequences corresponding to two consecutive, homologous 71 bp exons, 9 and 10, in the α (I) subunit gene (Helaakoski et al., 1989, 1994).

The mRNAs for the α (I) and α (II) subunits, and also the corresponding polypeptides, are expressed in a variety of human and mouse cells and tissues (Helaakoski et al., 1995; Annunen et al., 1997; P. Annunen and K. I. Kivirikko, unpublished). The type I enzyme accounts for 60–90% of the total prolyl 4-hydroxylase activity in many cell types and tissues and the type II enzyme for 10–40%, but the opposite situation exists in cultured chondrocytes and cartilage, in which the type II enzyme represents about 70–80% (P. Annunen and K. I. Kivirikko, unpublished).

Molecular cloning and characterization of the β subunit of human prolyl 4-hydroxylase indicated, surprisingly, that this polypeptide is identical to another enzyme, protein disulfide isomerase (PDI, EC 5.3.4.1), and has PDI activity even when present in the native prolyl 4-hydroxylase tetramer (Koivu et al., 1987; Pihlajaniemi et al., 1987). The PDI polypeptide is synthesized in a large excess over the α subunits, and thus regulation of the amounts of active prolyl 4-hydroxylase occurs mainly through the latter (Kivirikko et al., 1989; Kivirikko et al., 1992). The multifunctional PDI polypeptide will be discussed further toward the end of this review.

An α subunit of prolyl 4-hydroxylase has also been cloned from the nematode *Caenorhabditis elegans* (Veijola et al., 1994). This α subunit forms an active recombinant enzyme in insect cells with either the *C. elegans* or human PDI polypeptide, but surprisingly, the enzymes containing the *C. elegans* α subunit are $\alpha\beta$ dimers (Fig. 1; Veijola et al., 1994, 1996a). The *C. elegans* PDI polypeptide also forms active prolyl 4-hydroxylases with human α subunits, but the resulting enzymes are $\alpha_2\beta_2$ tetramers (Veijola et al., 1996a). Formation of a tetramer or dimer thus depends on the properties of the α subunit. It is still not known whether any of the other nonvertebrate prolyl 4-hydroxylases are dimers rather

than tetramers. It may be noted that some forms of prolyl 4-hydroxylase do not even have any PDI subunit (Fig. 1), as the enzymes from unicellular and multicellular green algae are α monomers (Kaska et al., 1988).

The processed *C. elegans* α subunit is 542 amino acids in size, the difference relative to the vertebrate α subunits (above) being mainly due to a 32-amino acid C-terminal extension (Fig. 2) which is not found in the vertebrate α subunits (Veijola et al., 1994). Deletion of this extension from the *C. elegans* α subunit prevents assembly of an enzyme dimer (Veijola et al., 1996a). The sequence of the *C. elegans* α subunit has features of both types of vertebrate α subunit (Veijola et al., 1994; Helaakoski et al., 1995), the degrees of amino acid sequence identity between the *C. elegans* α subunit and the human α (I) and α (II) subunits, excluding the C-terminal extension present only in the *C. elegans* α subunit, being 45% and 42%, respectively (Veijola et al., 1994; Annunen et al., 1997). The highest degree of identity between all these α subunits is found within their C-terminal regions (Fig. 2).

Peptide Substrates and Corresponding Inhibitors

Prolyl 4-hydroxylase is located within the lumen of the endoplasmic reticulum and acts *in vivo* on proline residues in appropriate sequences of growing and newly synthesized polypeptide chains. The enzyme does not hydroxylate free proline or tripeptides with the structure Gly-X-Pro or Pro-Gly-X *in vitro*, whereas tripeptides with the structure X-Pro-Gly are hydroxylated. Studies with various peptides have indicated that the minimum sequence requirement for a vertebrate prolyl 4-hydroxylase is fulfilled by an -X-Pro-Gly- triplet (see Kivirikko et al., 1992). However, the tetrapeptides Pro-Pro-Ala-Pro and Pro-Pro-Glu-Pro are also hydroxylated, although at a very low rate (Atreya et al., 1991). This agrees with the presence of a few single -X-4Hyp-Ala- sequences in the subcomponent C1q of complement and in some collagen polypeptide chains.

The K_m values for short peptides with a single hydroxylatable -X-Pro-Gly- unit are usually very high and decrease markedly with an increase in the number of repeated -X-Pro-Gly- triplets. The K_m of type I prolyl 4-hydroxylase for the tripeptide Pro-Pro-Gly, for example, is about 20 mM, whereas those for (Pro-Pro-Gly)₅ and (Pro-Pro-Gly)₂₀ are about 2 mM and 50 μ M, respectively, and that for nonhydroxylated type I procollagen polypeptide chains with molecular weights of about

150,000 is only about 0.2 μM , all these values being expressed as concentrations of the -X-Pro-Gly- units. This marked dependence of the K_m on the length of the collagenous sequence may explain why 4-hydroxyproline is found in only a very few proteins with a single -X-Pro-Gly- triplet (for references, see Kivirikko et al., 1992; Kivirikko and Pihlajaniemi, 1998).

The interaction of prolyl 4-hydroxylase with peptide substrates is further affected by the amino acid in the X position of the triplet to be hydroxylated, by other adjacent amino acids and by the peptide conformation (see Kivirikko et al., 1992). The triple-helical conformation completely prevents hydroxylation, indicating that hydroxylation of the newly synthesized collagenous polypeptide chains in cells must take place before triple helix formation. It has been proposed that the X-Pro segment may have to assume a poly(L-proline) II conformation at the binding site of the enzyme, while the Pro-Gly segment may have to assume a β turn for the proline residue to be hydroxylated at the catalytic site (Atreya et al., 1991). No direct data, however, are currently available to support this model.

Poly(L-proline) is a highly effective competitive inhibitor of the vertebrate type I prolyl 4-hydroxylase (Fig. 1), the K_i decreasing with increasing chain length, so that poly(L-proline) with a molecular weight of 15,000 has a K_i of 0.02 μM . In the cases of plant prolyl 4-hydroxylases, poly(L-proline) is an effective polypeptide substrate (see Kivirikko et al., 1992). The vertebrate type II enzyme differs from both these prolyl 4-hydroxylases in that poly(L-proline) is only a very weak inhibitor (Fig. 1), its K_i values being up to 1000 times higher than those of the type I enzyme (Helaakoski et al., 1995; Annunen et al., 1997). The *C. elegans* prolyl 4-hydroxylase dimer resembles the vertebrate type II enzyme in this respect (Veijola et al., 1994, 1996a). These findings suggest that there must be some distinct differences in the structures of the peptide binding sites between the various forms of prolyl 4-hydroxylase. Comparison of K_m values of the vertebrate type I and type II enzymes for three peptide substrates, Gly-Val-Pro-Val-Gly, (Pro-Pro-Gly)₁₀ and nonhydroxylated type I procollagen, has in fact indicated that these values are about 3–6-fold in the case of the type II enzyme, but this difference is very minor when compared with the up to 1000-fold difference in the K_i values for poly(L-proline) (Annuen et al., 1997).

Peptides in which the proline residue to be hydroxylated has been replaced by 5-oxaproline, a proline analog containing oxygen as part of the five-membered ring, are suicide inactivators of prolyl 4-hydroxylases (Gün-

zler et al., 1988a). The most effective such peptide studied to date has the structure benzyloxycarbonyl-Phe-Oxaproline-Gly-benzylester and inactivates type I prolyl 4-hydroxylase by 50% in 1 h at a 0.8 μM concentration. Replacement of the glycine by other amino acids completely eliminates the inactivating effect, while replacement of the phenylalanine by alanine, valine or glutamate reduces its potency in this order (Günzler et al., 1988a).

Cosubstrates and Reaction Mechanisms

Prolyl 4-hydroxylase requires Fe^{2+} , 2-oxoglutarate, O_2 and ascorbate (Fig. 3), there being no differences in the K_m values for these cosubstrates between the various enzyme forms (Veijola et al., 1994, 1996a; Helaakoski et al., 1995; Annunen et al., 1997). The 2-oxoglutarate is stoichiometrically decarboxylated during hydroxylation, with one atom of the O_2 molecule being incorporated into the succinate and the other into the hydroxy group formed on the proline residue. Ascorbate is not consumed stoichiometrically, and the enzyme can complete a number of catalytic cycles at an essentially maximal rate in its absence (Fig. 3A). The reaction requiring

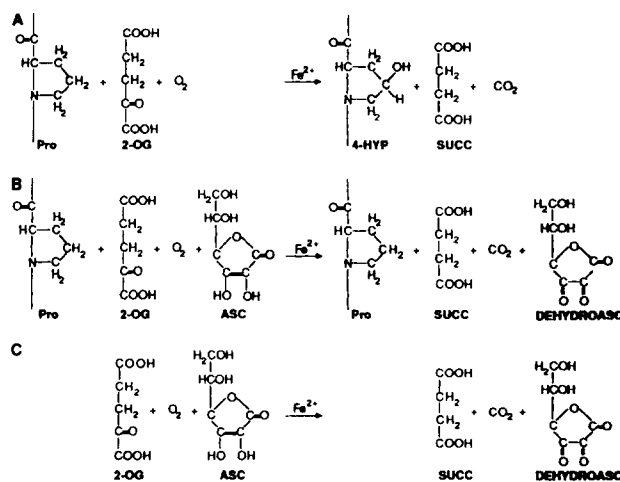


Figure 3. Schematic representation of the reactions catalyzed by prolyl 4-hydroxylases. The 2-oxoglutarate is stoichiometrically decarboxylated during the hydroxylation reaction, which does not need ascorbate (A). The enzyme also catalyzes an uncoupled decarboxylation of 2-oxoglutarate without subsequent hydroxylation of the peptide substrate. Ascorbate is stoichiometrically consumed in this uncoupled reaction, which may occur in either the presence (B) or absence (C) of the peptide substrate. Pro, proline; 4-Hyp, 4-hydroxyproline; 2-Og, 2-oxoglutarate; Succ, succinate; Asc, ascorbate; Dehydroasc, dehydroascorbate. Reproduced from Kivirikko et al. (1989).

ascorbate is an uncoupled decarboxylation of 2-oxoglutarate, i.e., decarboxylation without subsequent hydroxylation (Fig. 3B, 3C).

Kinetic studies (Myllylä et al., 1977), theoretical considerations (Hanauske-Abel and Günzler, 1982; Hanauske-Abel, 1991) and extensive site-directed mutagenesis studies (Lamberg et al., 1995; Myllyharju and Kivirikko, 1997) have made it possible to elucidate the reaction mechanism and structural features of the catalytic sites. The catalytic cycle can be divided into two half-reactions: initial generation of the hydroxylating species, and its subsequent utilization for 4-hydroxyproline formation (Hanauske-Abel and Günzler, 1982; Hanauske-Abel, 1991).

The current model for a catalytic site assumes that the Fe^{2+} is located in a pocket coordinated with the enzyme by three side-chains (Hanauske-Abel and Günzler, 1982). This pocket may be highly hydrophobic, as has recently been demonstrated for a related enzyme, isopenicillin N synthase (Roach et al., 1995). Site-directed mutagenesis studies and other data indicate that these three ligands in the human $\alpha(\text{I})$ subunit (Fig. 4) are histidines 412 and 483 and aspartate 414 (Myllylä et al., 1992; Lamberg et al., 1995; Myllyharju and Kivirikko, 1997). Mutation of any of these residues to serine or

alanine inactivates the enzyme completely, with no inhibition of tetramer assembly or binding of the tetramer to poly(L-proline). Mutation of aspartate 414 to glutamate increases the K_m for Fe^{2+} about 15-fold with no change in the V_{\max} of the reaction (Myllyharju and Kivirikko, 1997). All three residues are conserved in the sequences of all forms of the α subunit determined so far (Fig. 2). Moreover, site-directed mutagenesis studies on a closely related enzyme, lysyl hydroxylase, have indicated that the three Fe^{2+} binding ligands in that enzyme are likewise two histidines and one aspartate located in corresponding positions (Pirskanen et al., 1996). Determination of the crystal structure of isopenicillin N synthase has further indicated that its three Fe^{2+} binding ligands are two histidines and one aspartate in corresponding positions (Roach et al., 1995, 1997). The Fe^{2+} in isopenicillin N synthase possesses between catalytic cycles two water molecules and a fourth protein ligand, a glutamine residue, which is not conserved through other members of the family of related enzymes (Roach et al., 1995). It thus seems probable that the Fe^{2+} of prolyl 4-hydroxylase likewise contains water molecules and an additional protein ligand occupying coordination sites involved in the subsequent binding of the 2-oxoglutarate and O_2 molecules.

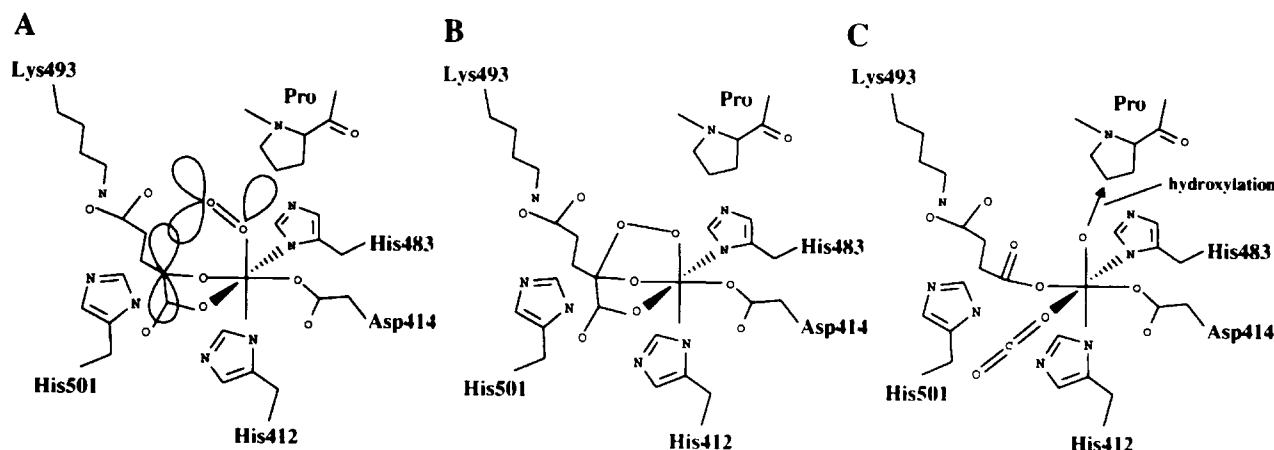


Figure 4. Schematic representation of the first half-reaction of prolyl 4-hydroxylase that takes place within the coordination sphere of the catalytic site Fe^{2+} and the critical residues at the cosubstrate binding sites. The Fe^{2+} is coordinated with the enzyme by three residues, His412, Asp414 and His483. The 2-oxoglutarate-binding site can be divided into two main subsites; subsite I consists of Lys493, which ionically binds the C5 carboxyl group of 2-oxoglutarate, while subsite II consists of two cis-positioned equatorial coordination sites of enzyme-bound Fe^{2+} and is chelated by the C1 carboxyl and C2 oxo functions. Molecular oxygen is assumed to be bound end-on in an axial position, producing a dioxygen unit. (A) One of the electron-rich orbitals of the dioxygen is directed to the electron-depleted orbital at the C2 of the 2-oxoglutarate bound to the iron. (B) Nucleophilic attack on C2 generates a tetrahedral intermediate, with loss of the double bond in the dioxygen unit and of double bond characteristics in the oxo-acid moiety. (C) Elimination of CO_2 coincides with the formation of succinate and a ferryl ion, which hydroxylates a proline residue in the peptide substrate in the second half-reaction. His501 is an additional important residue at the catalytic site, probably being involved in both coordination of the C1 carboxyl group of 2-oxoglutarate to Fe^{2+} and cleavage of the tetrahedral ferryl intermediate. Modified from Hanauske-Abel (1991) and Myllyharju and Kivirikko (1997).

The 2-oxoglutarate binding site can be divided into three distinct subsites (Hanauske-Abel and Günzler, 1982; Majamaa et al., 1984). Subsite I is a positively charged residue of the enzyme that ionically binds the C5 carboxyl group of 2-oxoglutarate; subsite II consists of two cis-positioned coordination sites of the enzyme-bound Fe^{2+} and is chelated by the C1-C2 moiety; while subsite III involves a hydrophobic binding site in the C3-C4 region of the cosubstrate. Site-directed mutagenesis studies (Myllyharju and Kivirikko, 1997) indicate that the residue forming subsite I in the human $\alpha(\text{I})$ subunit is lysine 493 (Fig. 4). Mutation of this residue to alanine or histidine inactivates the enzyme completely, whereas mutation to arginine inactivates it by 85% under standard assay conditions, the main effect being an increase of about 15-fold of the K_m for 2-oxoglutarate with an unaltered V_{\max} .

Molecular oxygen is assumed to be bound to the Fe^{2+} end-on in an axial position, producing a species characterized by doubly occupied orbitals, the dioxygen unit (Hanauske-Abel and Günzler, 1982). One of the electron-rich orbitals of the dioxygen unit is directed to the electron-depleted orbital at the C2 of the 2-oxoglutarate bound to the iron (Fig. 4A), so that the C2 undergoes rehybridization from its sp^2 hybridized planar oxo structure to an sp^3 hybridized tetrahedral transition state and forms a covalent bond with the noncoordinated atom of the dioxygen unit (Fig. 4B). This weakens both the C-C bond in the 2-oxoglutarate and the O-O bond in the dioxygen (Hanauske-Abel and Günzler, 1982; Hanauske-Abel, 1991). Decarboxylation will then take place simultaneously with the cleavage of the O-O bond, and the original C2 of 2-oxoglutarate, which has become the C1 of succinate, returns to the sp^2 hybridization. At the same time, a ferryl ion is formed (Fig. 4C), which hydroxylates a proline residue in the peptide substrate in the second half-reaction (Hanauske-Abel and Günzler, 1982; Hanauske-Abel, 1991). This half-reaction renews the enzyme-bound Fe^{2+} and concludes the catalytic cycle.

Histidine 501 is an additional critical residue at the catalytic site (Fig. 4). Detailed studies on various histidine 501 mutants have indicated that this residue probably has two functions. It directs the orientation of the C1 carboxyl group of 2-oxoglutarate to the active iron center, and it accelerates the breakdown of the tetrahedral ferryl intermediate (Fig. 4B) to succinate, CO_2 and ferryl ion (Myllyharju and Kivirikko, 1997).

Ascorbate is not needed in the hydroxylation reaction but is consumed stoichiometrically with the decarboxylation of 2-oxoglutarate in the uncoupled reaction (de

Jong and Kemp, 1984; Myllylä et al., 1984). In such reaction cycles (Fig. 3C), the ferryl ion is probably converted to Fe^{3+}O^- , making prolyl 4-hydroxylase unavailable for new reaction cycles until reduced by ascorbate. The enzyme catalyzes uncoupled reaction cycles even in the presence of saturating concentrations of its peptide substrates (Fig. 3B), and thus the main biological function of ascorbate in the prolyl 4-hydroxylase reaction, both *in vitro* and *in vivo*, is probably that of acting as an alternative oxygen acceptor in the uncoupled catalytic cycles (Myllylä et al., 1984). The ascorbate binding site also contains the two cis-positioned equatorial coordination sites of the enzyme-bound iron discussed above, and is thus partially identical to the binding site of 2-oxoglutarate (Majamaa et al., 1986). Modifications of the ring atoms of ascorbate that are needed for binding to iron abolish the cosubstrate activity, whereas many derivatives that differ only in their side chain are fully active (Majamaa et al., 1986; Tschank et al., 1994).

Nonpeptide Inhibitors

Inhibitors of prolyl 4-hydroxylase have now been characterized with respect to all its cosubstrates. In recent years, interest has been focused especially on competitive inhibitors with respect to 2-oxoglutarate (e.g., Majamaa et al., 1984; Cunliffe and Franklin, 1986; Cunliffe et al., 1992; Baader et al., 1994). Pyridine 2,4-dicarboxylate and pyridine 2,5-dicarboxylate (Fig. 5), for example, have functional groups that can interact at all the subsites of the 2-oxoglutarate binding site and inhibit the enzyme with K_i values of 2 μM and 0.8 μM , respectively (Hanauske-Abel and Günzler, 1982; Majamaa et al., 1984). These two compounds are only very weak inhibitors of 2-oxoglutarate dehydrogenase, an enzyme that differs from prolyl 4-hydroxylase in that its reaction mechanism does not involve any metal ion. Variation of the structures of these compounds has indicated that the K_i increases substantially if the chelating moiety is destroyed by omitting or shifting the C2 carboxyl group or by substitution of the aromatic nitrogen (Majamaa et al., 1984). A similar effect is seen if the carboxyl group believed to be bound at subsite I is omitted or shifted to a position other than 4 or 5 (Majamaa et al., 1984). The C2 or C5 group of pyridine 2,5-dicarboxylate can also be replaced by several other groups with a retention or improvement in potency (Tucker and Thomas, 1992; Dowell et al., 1993). It has been pointed out, however, that pyridine 2,5-dicarboxylate and 2-oxoglutarate may bind to prolyl 4-hydroxylase in different ways, and thus

the data concerning modifications of this compound must be interpreted with caution (Cunliffe et al., 1992).

N-oxalylglycine (Fig. 5) has a K_i of about 0.5–8 μM (Cunliffe et al., 1992; Baader et al., 1994). This compound, which differs from 2-oxoglutarate only by substitution of -NH- for the methylene group at C3, does not act as a cosubstrate, however. This agrees with the reaction mechanism discussed above, in which the decarboxylation involves a nucleophilic attack by an electron pair in the dioxygen unit on the electron-deficient orbital at the C2 of 2-oxoglutarate. The C3 in 2-oxoglutarate is sp^3 hybridized and cannot contribute any electron density to the p_z orbital of C2, whereas the C3 in oxalylglycine is replaced by the sp^2 hybridized nitrogen atom (Baader et al., 1994), the p_z orbital of which has an electron pair that can contribute to the electron density of C2, and therefore a nucleophilic attack is no longer possible (Baader et al., 1994).

3,4-Dihydroxybenzoate (Fig. 5) also has functional groups needed for binding at all the subsites of the 2-oxoglutarate binding site and inhibits the enzyme with a K_i of 5 μM (Majamaa et al., 1986). It nevertheless differs from the pyridine derivatives in being competitive with respect to both 2-oxoglutarate and ascorbate, whereas the latter are uncompetitive with respect to ascorbate. This agrees with the concept that the ascorbate binding

site is partially identical to the binding site of 2-oxoglutarate. The dihydroxybenzoate and pyridine compounds probably become bound to different enzyme forms, as determined by the oxidation state of the enzyme-bound iron (Majamaa et al., 1986).

L-Mimosine (Fig. 5) inhibits prolyl 4-hydroxylase by 50% at a 120 μM concentration (McCaffrey et al., 1995), and also causes a reversible, dose-dependent inhibition of DNA synthesis and the hydroxylation of deoxyhypusine to hypusine, an amino acid needed by the initiation factor eIF-5A and cell cycle transition. Thus a single compound inhibits both deoxyhypusyl hydroxylase and prolyl 4-hydroxylase, leading to both antiproliferative and fibrosuppressive effects (McCaffrey et al., 1995).

Three groups of compounds have also been identified that act as irreversible inactivators of prolyl 4-hydroxylase, probably by a suicide mechanism. The first group consists of peptides containing 5-oxaproline, as discussed above. The second group includes coumalic acid (Fig. 5), which acts as a 2-oxoglutarate analog but is only a weak inactivator, as it lacks a functional group needed to bind to the Fe^{2+} at a catalytic site (Günzler et al., 1987). The third group consists of the anthracyclines doxorubicin (Fig. 5) and daunorubicin, which inactivate prolyl 4-hydroxylase by 50% in 1 h at a 60 μM concentration (Günzler et al., 1988b). This inactivation can be prevented by high concentrations of ascorbate or low concentrations of its competitive analogs, whereas 2-oxoglutarate and its analogs offer no protection (Günzler et al., 1988b).

Many of these inhibitors show low membrane permeability, which makes them ineffective in cultured cells and *in vivo*. Further modifications of the compounds can be used to overcome this problem, however. A lipophilic proinhibitor derivative pyridine 2,4-dicarboxylic acid di(methoxyethyl)amide (HOE 077, Hoechst; Hanauske-Abel, 1991), for example selectively inhibits collagen accumulation in the liver of rats in two models of hepatic fibrosis (Bickel et al., 1991; Böker et al., 1991). It thus seems possible to develop prolyl 4-hydroxylase inhibitors into antifibrotic drugs, but more effective compounds are needed before this approach can be used for the treatment of patients with fibrotic diseases.

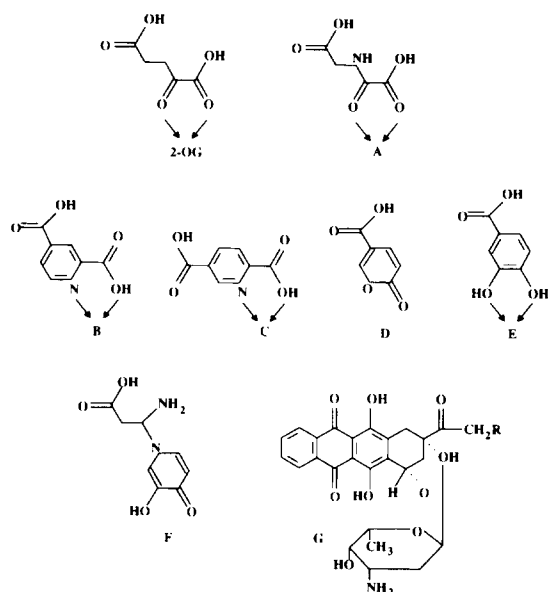


Figure 5. Structures of certain nonpeptide inhibitors of prolyl 4-hydroxylase. (A) N-oxalylglycine, (B) pyridine 2,4-dicarboxylate, (C) pyridine 2,5-dicarboxylate, (D) coumalic acid, (E) 3,4-dihydroxybenzoate, (F) L-mimosine and (G) doxorubicin (2-OG) 2-oxoglutarate.

The Multifunctional PDI Polypeptide

PDI, an abundant protein within the lumen of the endoplasmic reticulum, catalyzes disulfide bond formation

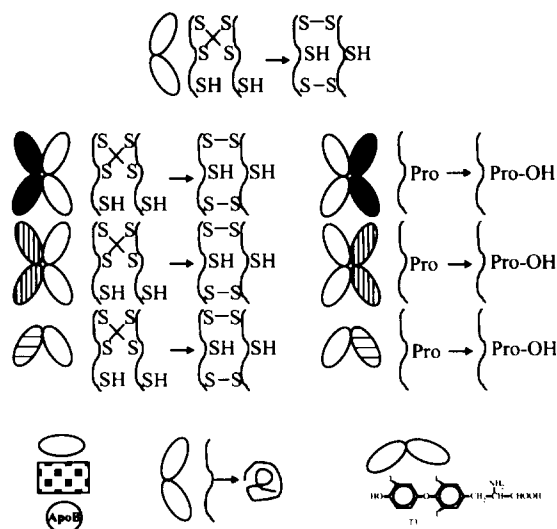


Figure 6. Schematic representation of some of the functions of the multifunctional PDI polypeptide. PDI catalyzes disulfide bond formation and thiol-disulfide interchange both when present as the homodimer and when present as the β subunit in the prolyl 4-hydroxylase tetramer. PDI also serves as the smaller subunit in the microsomal triglyceride transfer protein dimer, as a chaperone-like polypeptide that probably assists the folding of many newly synthesized proteins, and as a major cellular thyroid hormone (T3) binding protein.

in the *in vivo* biosynthesis of various secretory and cell surface proteins, including the collagens. PDI also catalyzes thiol-disulfide interchange *in vitro*, leading to net protein disulfide formation, reduction or isomerization, depending on the reaction conditions (see Freedman et al., 1994; Kivirikko and Pihlajaniemi, 1998).

PDI is a multifunctional polypeptide (Fig. 6) which serves as the β subunit in all forms of animal prolyl 4-hydroxylases (see above) and in the microsomal triglyceride transfer protein dimer (Wetterau et al., 1990), and as a chaperone-like polypeptide that binds various peptides within the lumen of the endoplasmic reticulum (Noiva et al., 1993), and probably assists in the folding of a number of newly synthesized proteins (LaMantia and Lennarz, 1993; Cai et al., 1994; Otsu et al., 1994; Puig et al., 1994; Rupp et al., 1994; Hayano et al., 1995; Yao et al., 1997). PDI is also a major cellular thyroid hormone-binding protein (Cheng et al., 1987), but the physiological significance of this property is unknown, and it may be related to the peptide-binding function. Still further reported functions are as a major phosphoprotein of the endoplasmic reticulum (Quémeur et al., 1994) that may undergo ATP-dependent autophosphorylation (Guthapfel et al., 1996), as a calcium

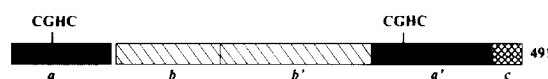


Figure 7. Schematic representation of the domain structure of the human PDI polypeptide. The two CGHC sequences indicate the two catalytic sites for PDI activity located in the two thioredoxin-like domains *a* and *a'*. Domains *b* and *b'* probably also have the thioredoxin fold, while domain *c* is a highly acidic, putative Ca^{2+} -binding region. Modified from Kemmink et al. (1997) and Kivirikko and Pihlajaniemi (1998).

binding protein (Lebeche et al., 1994), as a dehydroascorbate reductase (Wells et al., 1990), and several less-well-characterized functions (see Kivirikko and Pihlajaniemi, 1998).

The PDI polypeptide has been cloned from many animal species, yeast, micro-organisms and plants (see Freedman et al., 1994; Kivirikko and Pihlajaniemi, 1998). The processed human PDI polypeptide consists of 491 amino acids and is synthesized in a form having a signal peptide of 17 additional residues (Pihlajaniemi et al., 1987). The polypeptide is a modular protein (Fig. 7) built up of domains *a*, *b*, *b'*, *a'* and *c* (Edman et al., 1985; Pihlajaniemi et al., 1987). The existence of an additional domain *e*, proposed as being located between domains *a* and *b*, is now very unlikely (Kemmink et al. 1995, 1996; Darby et al., 1996). The *a* and *a'* domains are similar in sequence to thioredoxin, and both contain the -Cys-Gly-His-Cys- motif, which represent two independently acting catalytic sites for PDI activity (Hawkins and Freedman, 1991; Vuori et al., 1992a; LaMantia and Lennarz, 1993). NMR characterization of the human domain *a* expressed in *E. coli* indicates that it also has similar patterns of secondary structure and β sheet topology to thioredoxin (Kemmink et al., 1995). Recent NMR studies on domain *b* and preliminary data on domain *b'* suggest that these may likewise have the thioredoxin fold, even though they have no catalytic site sequences and show no significant amino acid sequence similarity to thioredoxin (Kemmink et al., 1997). Thus the main part of the PDI polypeptide appears to consist of two catalytically active and two inactive thioredoxin modules (Kemmink et al., 1997).

Domain *c*, which represents a putative Ca^{2+} binding region (Lebeche et al., 1994), is rich in acidic amino acids, 18 out of the 29 extreme C-terminal residues in the human polypeptide being either aspartate or glutamate. The C-terminus contains the -Lys-Asp-Glu-Leu motif or a functional variant of it, which is both necessary and sufficient for the retention of a polypeptide

within the lumen of the endoplasmic reticulum (Pelham, 1990). Deletion of this sequence from the PDI polypeptide led to the secretion of considerable amounts of both the free polypeptide and an active prolyl 4-hydroxylase tetramer from insect cells expressing a recombinant human prolyl 4-hydroxylase (Vuori et al., 1992c). Thus one function of the PDI subunit in prolyl 4-hydroxylases is to retain the enzyme within the lumen of the endoplasmic reticulum.

When a prolyl 4-hydroxylase tetramer or the microsomal triglyceride transfer protein dimer is dissociated by various means, the corresponding α subunit immediately forms insoluble aggregates with no prolyl 4-hydroxylase (see Kivirikko et al., 1992) or lipid transfer (Wetterau et al., 1991) activity. Also, when a recombinant prolyl 4-hydroxylase (Vuori et al., 1992b; Veijola et al., 1994; Helaakoski et al., 1995) or microsomal triglyceride transfer protein (Lamberg et al., 1996b) α subunit is expressed in insect cells alone, without the PDI polypeptide, it forms insoluble, inactive aggregates. The prolyl 4-hydroxylase α subunit similarly forms aggregates when expressed in a cell-free translation/translocation system of dog pancreas microsomes depleted of PDI and other soluble proteins of the endoplasmic reticulum (John et al., 1993). Thus one function of the PDI polypeptide in prolyl 4-hydroxylases and the microsomal triglyceride transfer protein is to keep the corresponding α subunits in a nonaggregated conformation. Experiments with a double mutant PDI polypeptide in which both catalytic sites had been inactivated by converting their sequences to -Ser-Gly-His-Cys- demonstrated that the disulfide isomerase activity of the polypeptide is not required for assembly of the prolyl 4-hydroxylase tetramer (Vuori et al., 1992c) or the microsomal triglyceride transfer protein dimer (Lamberg et al., 1996b); neither is it required for prolyl 4-hydroxylase activity or lipid transfer activity of the corresponding protein. Coexpression experiments have further demonstrated that the PDI polypeptide cannot be replaced by either ERp60 (Koivunen et al., 1996) or a *C. elegans* PDI isoform (Veijola et al., 1996a), which both belong to the family of PDI-related polypeptides (Freedman et al., 1994; Kivirikko and Pihlajaniemi, 1998) and contain two thioredoxin-like domains with active catalytic site sequences.

The chaperone BiP forms soluble complexes with the prolyl 4-hydroxylase α subunits in coexpression experiments in insect cells (Veijola et al., 1996b) and probably also in normal biosynthesis of the tetramer (John and Bulleid, 1996). Soluble α subunit-BiP complexes have no prolyl 4-hydroxylase activity, however, indicating that

the function of the PDI polypeptide is not only that of keeping the α subunits in solution but is more specific, probably that of keeping them in a catalytically active, nonaggregated conformation (Veijola et al., 1996b).

This function of the PDI polypeptide in prolyl 4-hydroxylases and the microsomal triglyceride transfer protein resembles that of some molecular chaperones such as Hsp90 in other proteins (Vuori et al., 1992b; Freedman et al., 1994). Site-directed mutagenesis studies have indicated that the extreme C terminal α helix of domain α' is critical for the roles of the PDI polypeptide both as a subunit and as a chaperone, thus supporting the view that these two functions may be related (P. Koivunen, P. Karvonen, T. Helaakoski, H. Notbohm and K. I. Kivirikko, unpublished).

Production of a Stable Prolyl 4-Hydroxylase Tetramer *in vivo* Requires Expression of Collagen Polypeptide Chains

Recent studies of the expression of the recombinant human prolyl 4-hydroxylase $\alpha(I)$ subunit and PDI polypeptide in the yeast *Pichia pastoris*, which has no endogenous prolyl 4-hydroxylase or collagen synthesis, have indicated that only a minor fraction of the polypeptides expressed are found in the form of the enzyme tetramer, whereas the vast majority are present in unassembled forms (Vuorela et al., 1997). Coexpression of these polypeptides with recombinant human type III collagen led to a marked increase in the tetramer assembly level, with no increase in the total amounts of the α subunit or the PDI polypeptide (Vuorela et al., 1997). The most likely mechanism is that the tetramer formed in the absence of collagen synthesis became rapidly dissociated back to its subunits, whereas coexpression with collagen stabilized the tetramer. Preliminary data suggest that the same phenomenon is also seen in insect cells, although their endogenous collagen synthesis partly prevents the dissociation which takes place in the absence of recombinant collagen synthesis (J. Myllyharju, A. Lamberg and K.I. Kivirikko, unpublished). It thus seems probable that this property of the enzyme may be found in all cell types.

As prolyl 4-hydroxylase in the absence of its peptide substrate catalyzes an uncoupled decarboxylation of 2-oxoglutarate (Fig. 3C), dissociation of the tetramer in the absence of collagen synthesis will prevent the enzyme from performing a reaction that consumes 2-oxoglutarate, O_2 and ascorbate within the lumen of the endoplasmic reticulum. This mechanism is likely to be partic-

ularly important in situations in which collagen synthesis is rapidly downregulated, as an active tetramer would otherwise continue to exist for a long time. Collagen synthesis thus appears to involve a control mechanism which is highly unusual in that production of a stable prolyl 4-hydroxylase tetramer requires expression of collagen polypeptide chains, while production of collagen molecules with stable triple helices requires expression of active prolyl 4-hydroxylase (Vuorela et al., 1997).

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References

- Annunen, P., Helaakoski, T., Myllyharju, J., Veijola, J., Pihlajaniemi, T. and Kivirikko, K.I.: Cloning of the human prolyl 4-hydroxylase α -subunit isoform α (II) and characterization of the type II enzyme tetramer. The α (I) and α (II) subunits do not form a mixed α (I) α (II) β_2 tetramer. *J. Biol. Chem.* 272: 17342–17348, 1997.
- Atreya, P.L. and Ananthanarayanan, V.S.: Interaction of prolyl 4-hydroxylase with synthetic peptide substrates. A conformational model for collagen proline hydroxylation. *J. Biol. Chem.* 266: 2852–2858, 1991.
- Baader, E., Tschank, G., Baringhaus, K.-H., Burghard, H. and Günzler, V.: Inhibition of prolyl 4-hydroxylase by oxalyl acid derivatives *in vitro*, in isolated microsomes and in embryonic chicken tissues. *Biochem. J.* 300: 525–530, 1994.
- Bassuk, J.A., Kao, W.W.-Y., Herzer, P., Kedersha, N.L., Seyer, J., DeMartino, J.A., Daugherty, B.L., Mark, G.E., III and Berg, R.A.: Prolyl 4-hydroxylase: Molecular cloning and the primary structure of the α -subunit from chicken enzyme. *Proc. Natl. Acad. Sci. USA* 86: 7382–7386, 1989.
- Bickel, M., Baader, E., Brocks, D.G., Engelbart, K., Günzler, V., Schmidts, H.L. and Vogel, G.H.: Beneficial effects of inhibitors of prolyl 4-hydroxylase in CCl₄-induced fibrosis of the liver in rats. *J. Hepatol.* 13: S26–S34, 1991.
- Böcker, K., Schwarting, G., Kaule, G., Günzler, V. and Schmidt, E.: Fibrosis of the liver in rats induced by bile duct ligation. Effects of inhibition of prolyl 4-hydroxylase. *J. Hepatol.* 13: S35–S40, 1991.
- Cai, H., Wang, C.-C. and Tsou, C.-L.: Chaperone-like activity of protein disulfide isomerase in the refolding of a protein with no disulfide bonds. *J. Biol. Chem.* 269: 24550–24552, 1994.
- Cheng, S.-Y., Gong, Q.-H., Parkison, C., Robinson, E.A., Appella, E., Merlino, G.T. and Pastan, I.: The nucleotide sequence of a human cellular thyroid hormone binding protein present in endoplasmic reticulum. *J. Biol. Chem.* 271: 2663–2666, 1987.
- Cunliffe, C.J. and Franklin, T.J.: Inhibition of prolyl 4-hydroxylase by hydroxyanthraquinones. *Biochem. J.* 239: 311–315, 1986.
- Cunliffe, C.J., Franklin, T.J., Hales, N.J. and Hill, G.B.: Novel inhibitors of prolyl 4-hydroxylase. 3. Inhibition by the substrate analogue N-oxaloglycine and its derivatives. *J. Med. Chem.* 35: 2652–2658, 1992.
- Darby, N.J., Kemmink, J. and Creighton, T.E.: Identifying and characterizing a structural domain of protein disulfide isomerase. *Biochemistry* 35: 10517–10528, 1996.
- de Jong, L. and Kemp, A.: Stoichiometry and kinetics of the prolyl 4-hydroxylase partial reaction. *Biochim. Biophys. Acta* 787: 105–111, 1984.
- Dowell, R.I., Hales, N.H. and Hadley, E.M.: Novel inhibitors of prolyl 4-hydroxylase. Part 4. Pyridine-2-carboxylic acid analogues with alternative 2-substituents. *Eur. J. Med. Chem.* 28: 513–516, 1993.
- Edman, J.C., Ellis, L., Blacher, R.W., Roth, R.A. and Rutter, W.J.: Sequence of protein disulfide isomerase and implications of its relationship to thioredoxin. *Nature* 317: 267–270, 1985.
- Freedman, R.B., Hirst, T.R. and Tuite, M.F.: Protein disulfide isomerase: building bridges in protein folding. *Trends Biochem. Sci.* 19: 331–336, 1994.
- Günzler, V., Brocks, D., Henke, S., Myllylä, R., Geiger, R. and Kivirikko, K.I.: Syncatalytic inactivation of prolyl 4-hydroxylase by synthetic peptides containing the unphysiologic amino acid 5-oxaproline. *J. Biol. Chem.* 263: 19498–19504, 1988a.
- Günzler, V., Hanauske-Abel, H.M., Myllylä, R., Kaska, D., Hanauske, A. and Kivirikko, K.I.: Syncatalytic inactivation of prolyl 4-hydroxylase by anthracyclines. *Biochem. J.* 251: 365–372, 1988b.
- Günzler, V., Hanauske-Abel, H.M., Myllylä, R., Mohr, J. and Kivirikko, K.I.: Time-dependent inactivation of chick embryo prolyl 4-hydroxylase by coumalic acid. Evidence for a syncatalytic mechanism. *Biochem. J.* 242: 163–169, 1987.
- Guthapfel, R., Guéguen, P. and Quéméneur, E.: ATP binding and hydrolysis by the multifunctional protein disulfide isomerase. *J. Biol. Chem.* 271: 2663–2666, 1996.
- Hanauske-Abel, H.M.: Prolyl 4-hydroxylase, a target enzyme for drug development. Design of suppressive agents and the *in vitro* effects of inhibitors and proinhibitors. *J. Hepatol.* 13: S8–S16, 1991.
- Hanauske-Abel, H.M. and Günzler, V.: A stereochemical concept for the catalytic mechanism of prolylhydroxylase. Applicability to classification and design of inhibitors. *J. Theor. Biol.* 94: 421–455, 1982.
- Hawkins, H.C. and Freedman, R.B.: The reactivities and ionization properties of the active-site dithiol groups of mammalian protein disulfide-isomerase. *Biochem. J.* 275: 335–339, 1991.
- Hayano, T. and Kikuchi, M.: Protein disulfide isomerase mutant lacking its isomerase activity accelerates protein folding in the cell. *FEBS Lett.* 372: 210–214, 1995.
- Helaakoski, T., Annunen, P., Vuori, K., MacNeil, I.A., Pihlajaniemi, T. and Kivirikko, K.I.: Cloning, baculovirus expression, and characterization of a second mouse prolyl 4-hydroxylase α -subunit isoform: Formation of an $\alpha_3\beta_2$ tetramer with the protein disulfide isomerase/ β subunit. *Proc. Natl. Acad. Sci. USA* 92: 4427–4431, 1995.
- Helaakoski, T., Veijola, J., Vuori, K., Rehn, M., Chow, L.T., Taillon-Miller, P., Kivirikko, K.I. and Pihlajaniemi, T.: Structure and expression of the human gene for the α subunit of prolyl 4-hydroxylase. The two alternatively spliced types of mRNA correspond to two homologous exons the sequences of which are expressed in a variety of tissues. *J. Biol. Chem.* 269: 27847–27854, 1994.

- Helaakoski, T., Vuori, K., Myllylä, R., Kivirikko, K.I. and Pihlajaniemi, T.: Molecular cloning of the α -subunit of human prolyl 4-hydroxylase: The complete cDNA-derived amino acid sequence and evidence for alternative splicing of RNA transcripts. *Proc. Natl. Acad. Sci. USA* 86: 4392–4396, 1989.
- Hopkinson, I., Smith, S.A., Donne, A., Gregory, H., Franklin, T.J., Grant, M.E. and Rosamund, J.: The complete cDNA derived sequence of the rat prolyl 4-hydroxylase α subunit. *Gene* 149: 391–392, 1994.
- John, D.C.A. and Bulleid, N.J.: Prolyl 4-hydroxylase: Defective assembly of α -subunit mutants indicates that assembled α -subunits are intramolecularly disulfide bonded. *Biochemistry* 33: 14018–14025, 1994.
- John, D.C.A. and Bulleid, N.J.: Intracellular dissociation and reassembly of prolyl 4-hydroxylase: the α -subunits associate with the immunoglobulin-heavy-chain binding protein (BiP) allowing reassembly with the β -subunit. *Biochem. J.* 317: 659–665, 1996.
- John, D.C.A., Grant, M.E. and Bulleid, N.J.: Cell-free synthesis and assembly of prolyl 4-hydroxylase: the role of the β -subunit (PDI) in preventing misfolding and aggregation of the α -subunit. *EMBO J.* 12: 1587–1592, 1993.
- Kaska, D.D., Myllylä, R., Günzler, V., Gibor, A. and Kivirikko, K.I.: Prolyl 4-hydroxylase from *Volvox carteri*. A low-M_r enzyme antigenically related to the α subunit of the vertebrate enzyme. *Biochem. J.* 256: 257–263, 1988.
- Kemmink, J., Darby, N.J., Dijkstra, K., Nilges, M. and Creighton, T.E.: Structure determination of the N-terminal thioredoxin-like domain of protein disulfide isomerase using multidimensional heteronuclear $^{13}\text{C}/^{15}\text{N}$ NMR spectroscopy. *Biochemistry* 35: 7684–7691, 1996.
- Kemmink, J., Darby, N.J., Dijkstra, K., Nilges, M. and Creighton, T.E.: The folding catalyst protein disulfide isomerase is constructed of active and inactive thioredoxin modules. *Curr. Biol.* 7: 239–245, 1997.
- Kemmink, J., Darby, N.J., Dijkstra, K., Scheek, R.M. and Creighton, T.E.: Nuclear magnetic resonance characterization of the N-terminal thioredoxin-like domain of protein disulfide isomerase. *Protein Sci.* 4: 2587–2593, 1995.
- Kivirikko, K.I., Myllylä, R. and Pihlajaniemi, T.: Protein hydroxylation: prolyl 4-hydroxylase, an enzyme with four co-substrates and a multifunctional subunit. *FASEB J.* 3: 1609–1617, 1989.
- Kivirikko, K.I., Myllylä, R. and Pihlajaniemi, T.: Hydroxylation of proline and lysine residues in collagens and other animal and plant proteins. In: *Post-Translational Modifications of Proteins*, ed. by Harding, J.J. and Crabbe, M.J.C., CRC Press, Boca Raton, pp. 1–51, 1992.
- Kivirikko, K.I. and Pihlajaniemi, T.: Collagen hydroxylases and the protein disulfide isomerase subunit of prolyl 4-hydroxylases. *Adv. Enzymol. Related Areas Mol. Biol.*, in press, 1998.
- Koivu, J., Myllylä, R., Helaakoski, T., Pihlajaniemi, T., Tasanen, K. and Kivirikko, K.I.: A single polypeptide acts both as the β subunit of prolyl 4-hydroxylase and as a protein disulfide-isomerase. *J. Biol. Chem.* 262: 6447–6449, 1987.
- Koivunen, P., Helaakoski, T., Annunen, P., Veijola, J., Räisänen, S., Pihlajaniemi, T. and Kivirikko, K.I.: ERp60 does not substitute for protein disulfide isomerase as the β -subunit of prolyl 4-hydroxylase. *Biochem. J.* 316: 599–605, 1996.
- LaMantia, M. and Lennarz, W.J.: The essential function of yeast protein disulfide isomerase does not reside in its isomerase activity. *Cell* 74: 899–908, 1993.
- Lamberg, A., Helaakoski, T., Myllyharju, J., Peltonen, S., Notbohm, H., Pihlajaniemi, T. and Kivirikko, K.I.: Characterization of human type III collagen expressed in a baculovirus system. Production of a protein with a stable triple helix requires coexpression with the two types of recombinant prolyl 4-hydroxylase subunit. *J. Biol. Chem.* 271: 11988–11995, 1996a.
- Lamberg, A., Jauhainen, M., Metso, J., Ehnholm, C., Shoulders, C., Scott, J., Pihlajaniemi, T. and Kivirikko, K.I.: The role of protein disulfide isomerase in the microsomal triglyceride transfer protein does not reside in its isomerase activity. *Biochem. J.* 315: 533–536, 1996b.
- Lamberg, A., Pihlajaniemi, T. and Kivirikko, K.I.: Site-directed mutagenesis of the α subunit of human prolyl 4-hydroxylase. Identification of three histidine residues critical for catalytic activity. *J. Biol. Chem.* 270: 9926–9931, 1995.
- Lebeche, D., Lucero, H.A. and Kaminer, B.: Calcium binding properties of rabbit liver protein disulfide isomerase. *Biochem. Biophys. Res. Commun.* 202: 556–561, 1994.
- Majamaa, K., Günzler, V., Hanauske-Abel, H.M., Myllylä, R. and Kivirikko, K.I.: Partial identity of the 2-oxoglutarate and ascorbate binding sites of prolyl 4-hydroxylase. *J. Biol. Chem.* 261: 7819–7823, 1986.
- Majamaa, K., Hanauske-Abel, H.M., Günzler, V. and Kivirikko, K.I.: The 2-oxoglutarate binding site of prolyl 4-hydroxylase. Identification of distinct subsites and evidence for 2-oxoglutarate decarboxylation in a ligand reaction at the enzyme-bound ferrous iron. *Eur. J. Biochem.* 138: 239–245, 1984.
- McCaffrey, T.A., Pomerantz, K., Sanborn, T.A., Spokojny, A., Du, B., Park, M.H., Folk, J.E., Lamberg, A., Kivirikko, K.I., Falcone, D.J., Mehta, S. and Hanauske-Abel, H.M.: Specific inhibition of eIF-5A and collagen hydroxylation by a single agent. Antiproliferative and fibrosuppressive effects on human coronary smooth muscle cells. *J. Clin. Invest.* 95: 446–455, 1995.
- Myllyharju, J. and Kivirikko, K.I.: Characterization of the iron- and 2-oxoglutarate-binding sites of human prolyl 4-hydroxylase. *EMBO J.* 16: 1173–1180, 1997.
- Myllyharju, J., Lamberg, A., Notbohm, H., Fietzek, P.P., Pihlajaniemi, T. and Kivirikko, K.I.: Expression of wild-type and modified pro α chains of human type I procollagen in insect cells leads to the formation of stable [α 1(I)]₂ [α 2(I)] heterotrimers and [α 1(I)]₃ homotrimers but not [α 2(I)]₃ homotrimers. *J. Biol. Chem.* 272: 21824–21830, 1997.
- Myllylä, R., Günzler, V., Kivirikko, K.I. and Kaska, D.D.: Modification of vertebrate and algal prolyl 4-hydroxylases and vertebrate lysyl hydroxylase by diethyl pyrocarbonate. Evidence for histidine residues in the catalytic site of 2-oxoglutarate-coupled dioxygenases. *Biochem. J.* 286: 923–927, 1992.
- Myllylä, R., Majamaa, K., Günzler, V., Hanauske-Abel, H.M. and Kivirikko, K.I.: Ascorbate is consumed stoichiometrically in the uncoupled reactions catalyzed by prolyl 4-hydroxylase and lysyl hydroxylase. *J. Biol. Chem.* 259: 5403–5405, 1984.
- Myllylä, R., Tuderman, L. and Kivirikko, K.I.: Mechanism of the prolyl hydroxylase reaction. 2. Kinetic analysis of the reaction sequence. *Eur. J. Biochem.* 80: 349–357, 1977.
- Noiva, R., Freedman, R.B. and Lennarz, W.J.: Peptide binding to protein disulfide isomerase occurs at a site distinct from the active site. *J. Biol. Chem.* 268: 19210–19217, 1993.
- Otsu, M., Omura, F., Yoshimori, T. and Kikuchi, M.: Protein disulfide isomerase associates with misfolded human lysozyme *in vivo*. *J. Biol. Chem.* 269: 6874–6877, 1994.

- Pelham, H.R.B.: The retention signal for soluble proteins of the endoplasmic reticulum. *Trends Biochem. Sci.* 15: 483–486, 1990.
- Pihlajaniemi, T., Helaakoski, T., Tasanen, K., Myllylä, R., Huh-tala, M.-L., Koivu, J. and Kivirikko, K.I.: Molecular cloning of the β -subunit of human prolyl 4-hydroxylase. This subunit and protein disulphide isomerase are products of the same gene. *EMBO J.* 6: 643–649, 1987.
- Pirskanen, A., Kaimio, A.-M., Myllylä, R. and Kivirikko, K.I.: Site-directed mutagenesis of human lysyl hydroxylase expressed in insect cells. Identification of histidine residues and an aspartic acid residue critical for catalytic activity. *J. Biol. Chem.* 271: 9398–9402, 1996.
- Puig, A., Lyles, M.M., Noiva, R. and Gilbert, H.F.: The role of the thiol/disulfide centers and peptide binding site in the chaperone and anti-chaperone activities of protein disulfide isomerase. *J. Biol. Chem.* 269: 19128–19135, 1994.
- Quéménéur, E., Guthapfel, R. and Guéguen, P.: A major phosphoprotein of the endoplasmic reticulum is protein disulfide isomerase. *J. Biol. Chem.* 269: 5485–5488, 1994.
- Roach, P.L., Clifton, I.J., Fülöp, V., Harlos, K., Barton, G.J., Hajdu, J., Andersson, I., Schofield, C.J. and Baldwin, J.E.: Crystal structure of isopenicillin N synthase is the first from a new structural family of enzymes. *Nature* 375: 700–704, 1995.
- Roach, P.L., Clifton, I.J., Hensgens, C.M.H., Shibata, N., Schofield, J., Hajdu, J. and Baldwin, J.E.: Structure of isopenicillin N synthase complexed with substrate and the mechanism of penicillin formation. *Nature* 387: 827–830, 1997.
- Rupp, K., Birnbach, U., Lundström, J., Nguyen Van, P. and Söling, H.-D.: Effects of CaBP2, the rat analog of ERp72, and of CaBP1 on the refolding of denatured reduced proteins. Comparison with protein disulphide isomerase. *J. Biol. Chem.* 269: 2501–2507, 1994.
- Tschank, G., Sanders, J., Baringhaus, K.H., Dallacker, E., Kivirikko, K.I. and Günzler, V.: Structural requirements for the utilization of ascorbate analogues in the prolyl 4-hydroxylase reaction. *Biochem. J.* 300: 75–79, 1994.
- Tucker, H. and Thomas, D.F.: Novel inhibitors of prolyl 4-hydroxylase. 2. 5-Amide substituted pyridine-2-carboxylic acids. *J. Med. Chem.* 35: 804–807, 1992.
- Veijola, J., Annunen, P., Koivunen, P., Page, A.P., Pihlajaniemi, T. and Kivirikko, K.I.: Baculovirus expression of two protein disulphide isomerase isoforms from *Caenorhabditis elegans* and characterization of prolyl 4-hydroxylases containing one of these polypeptides as their β subunit. *Biochem. J.* 317: 721–729, 1996a.
- Veijola, J., Koivunen, P., Annunen, P., Pihlajaniemi, T. and Kivirikko, K.I.: Cloning, baculovirus expression, and characterization of the α subunit of prolyl 4-hydroxylase from the nematode *Caenorhabditis elegans*. This α subunit forms an active $\alpha\beta$ dimer with the human protein disulfide isomerase/ β subunit. *J. Biol. Chem.* 269: 26746–26753, 1994.
- Veijola, J., Pihlajaniemi, T. and Kivirikko, K.I.: Coexpression of the α subunit of human prolyl 4-hydroxylase with BiP polypeptide in insect cells leads to the formation of soluble and insoluble complexes. Soluble α subunit-BiP complexes have no prolyl 4-hydroxylase activity. *Biochem. J.* 315: 613–618, 1996b.
- Wells, W.W., Xu, D.P., Yang, Y. and Rocque, P.A.: Mammalian thioltransferase (glutaredoxin) and protein disulfide isomerase have dehydroascorbate reductase activity. *J. Biol. Chem.* 265: 15361–15364, 1990.
- Wetterau, J.R., Combs, K.A., McLean, L.R., Spinner, S.N. and Aggerbeck, L.P.: Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. *Biochemistry* 30: 9728–9735, 1991.
- Wetterau, J.R., Combs, K.A., Spinner, S.N. and Joiner, B.J.: Protein disulfide isomerase is a component of the microsomal triglyceride transfer protein complex. *J. Biol. Chem.* 265: 9801–9807, 1990.
- Vuorela, A., Myllyharju, J., Nissi, R., Pihlajaniemi, T. and Kivirikko, K.I.: Assembly of human prolyl 4-hydroxylase and type III collagen in the yeast *Pichia pastoris*: formation of a stable enzyme tetramer requires coexpression with collagen and assembly of a stable collagen requires coexpression with prolyl 4-hydroxylase. *EMBO J.* 16: 6702–6712, 1997.
- Vuori, K., Myllylä, R., Pihlajaniemi, T. and Kivirikko, K.I.: Expression and site-directed mutagenesis of human protein disulfide-isomerase in *Escherichia coli*. This multifunctional polypeptide has two independently acting catalytic sites for the isomerase activity. *J. Biol. Chem.* 267: 7211–7214, 1992a.
- Vuori, K., Pihlajaniemi, T., Marttila, M. and Kivirikko, K.I.: Characterization of the human prolyl 4-hydroxylase tetramer and its multifunctional protein disulfide-isomerase subunit synthesized in a baculovirus expression system. *Proc. Natl. Acad. Sci. USA* 89: 7467–7470, 1992b.
- Vuori, K., Pihlajaniemi, T., Myllylä, R. and Kivirikko, K.I.: Site-directed mutagenesis of human protein disulphide isomerase: effect on the assembly, activity and endoplasmic reticulum retention of human prolyl 4-hydroxylase in *Spodoptera frugiperda* insect cells. *EMBO J.* 11: 4213–4217, 1992c.
- Yao, Y., Zhou, Y.-C. and Wang, C.-C.: Both the isomerase and chaperone activities of protein disulfide isomerase are required for the reactivation of reduced and denatured acidic phospholipase A2. *EMBO J.* 16: 651–658, 1997.

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