Collagen Prolyl 4-Hydroxylase Tetramers and Dimers Show Identical Decreases in $K_m$
Values for Peptide Substrates with Increasing Chain Length

MUTATION OF ONE OF THE TWO CATALYTIC SITES IN THE TETRAMER INACTIVATES THE ENZYME BY MORE THAN HALF*

Liisa Kukkola‡, Peppi Koivunen‡, Outi Pakkanen‡, Antony P. Page§, and Johanna Myllyharju‡¶

From the ‡Collagen Research Unit, Biocenter Oulu and Department of Medical Biochemistry and Molecular Biology, University of Oulu, FIN-90014 Oulu, Finland, and §Wellcome Centre for Molecular Parasitology, Anderson College, University of Glasgow, Glasgow G11 6NU, Scotland, United Kingdom.

Correspondence to Dr. Johanna Myllyharju, Department of Medical Biochemistry and Molecular Biology, P.O. Box 5000, University of Oulu, FIN-90014 Oulu, Finland
Tel: +358-8-537 5740, Fax: +358-8-537 5811
Email: johanna.myllyharju@oulu.fi

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The collagen prolyl 4-hydroxylases (collagen P4Hs, EC 1.14.11.2) play a key role in the synthesis of the extracellular matrix. The vertebrate enzymes are $\alpha_2\beta_2$ tetramers, the $\beta$ subunit being identical to protein disulfide isomerase (PDI). The main C. elegans collagen P4H form is an unusual PHY-1/PHY-2/(PDI)$_2$ mixed tetramer consisting of two types of catalytic $\alpha$ subunit, but the PHY-1 and PHY-2 polypeptides also form active PHY/PDI dimers. The lengths of peptide substrates have a major effect on their interaction with the P4H tetramers, the $K_m$ values decreasing markedly with increasing chain length. This phenomenon has been explained in terms of processive binding of the two catalytic subunits to long peptides. We determined here the $K_m$ values of a collagen P4H having two catalytic sites, the C. elegans mixed tetramer, and a form having only one such site, the PHY-1/PDI dimer, for peptides of varying lengths. All the $K_m$ values of the PHY-1/PDI dimer were found to be about 1.5-2.5 times those of the tetramer, but increasing peptide length led to identical decreases in the values of both enzyme forms. The $K_m$ for a nonhydroxylated collagen fragment with 33 -X-Y-Gly- triplets but only 11 -X-Pro-Gly- triplets was found to correspond to the number of the former rather than the latter. To study the individual roles of the two catalytic sites in a tetramer, we produced mutant PHY-1/PHY-2/(PDI)$_2$ tetramers in which binding of the Fe$^{2+}$ ion or 2-oxoglutarate to one of the two catalytic sites was prevented. The activities of the mutant tetramers decreased to markedly less than 50% of that of the wild-type, being about 5-10% and 20-30% with the enzymes having one of the two Fe$^{2+}$-binding sites or 2-oxoglutarate-binding sites inactivated, respectively, while the $K_m$ values for these cosubstrates or peptide substrates were not affected. Our data thus indicate that although collagen P4Hs do not act on peptide substrates by a processive mechanism, prevention of hydroxylation at one of the two catalytic sites in the tetramer impairs the function of the other catalytic site.
The collagen prolyl 4-hydroxylases (P4Hs) catalyze the formation of 4-hydroxyproline in -X-Pro-Gly- sequences in collagens and more than 20 proteins with collagen-like domains (1-5). This enzyme resident in the endoplasmic reticulum (ER) is essential for collagen synthesis, as the resulting 4-hydroxyproline residues are necessary for the formation of stable collagen triple helices (1-5). A novel family of cytoplasmic and nuclear P4Hs playing a critical role in the oxygen-dependent degradation of the hypoxia-inducible transcription factor HIFα has recently been identified (6-8). The HIF-P4Hs hydroxylate proline residues in -Leu-X-X-Leu-Ala-Pro-X- sequences (6-8).

Collagen P4Hs from all vertebrate sources studied are αβ2 tetramers in which the β subunit is identical to protein disulfide isomerase (PDI) (1-3). Three isoforms of the catalytic α subunit have been characterized from human and mouse sources and shown to form [α(I)]β2, [α(II)]β2 and [α(III)]β2 tetramers, called the type I, II and III enzymes, respectively (9-12). Insect cell coexpression studies have indicated that no mixed α(I)α(II)β2 tetramers are formed from the corresponding vertebrate subunits (10). The type I enzyme is the main form in most cell types and tissues, but the type II enzyme is a major form in chondrocytes, osteoblasts, endothelial cells and cells in epithelial structures (13, 14). The α(III) mRNA is expressed in many tissues, but at considerably lower levels than the α(I) and α(II) mRNAs (11, 12). Three Caenorhabditis elegans α subunit isoforms, PHY-1, PHY-2 and PHY-3, have also been characterized and shown to assemble into unique P4H forms (15-20). PHY-1 and PHY-2 are involved in the synthesis of nematode cuticle collagens (16, 17), whereas PHY-3 is likely to be involved in the synthesis of collagens in early embryos (19). PHY-1 and PHY-2 mainly assemble into a unique mixed PHY-1/PHY-2/(PDI-2)2 tetramer with the C. elegans PDI orthologue PDI-2, but can also form active PHY-1/PDI-2 and PHY-2/PDI-2 dimers, although very ineffectively (15, 20). Surprisingly, the PHY-1 polypeptide efficiently forms an active collagen P4H dimer with the human PDI (15).

The hydroxylation reaction catalyzed by P4Hs requires Fe²⁺, 2-oxoglutarate, O₂ and ascorbate and involves oxidative decarboxylation of 2-oxoglutarate (1-3). The $K_m$ values of the collagen P4Hs for peptide substrates decrease markedly with increasing chain length of the substrate (1-3). This finding has been explained by a processive mechanism of binding of
long peptide substrates (2, 21, 22). According to this model the enzyme-substrate complex is formed at one catalytic site upon the first encounter, and before the first hydroxylation is complete, the other catalytic site has a high chance of encountering another region of the peptide substrate (21, 22). Such a mechanism leads to a low $K_m$ for a long peptide by overcoming diffusional constraints on the association between the enzyme and the various substrate sites present in the peptide. The processive mechanism requires the presence of two active catalytic sites. The catalytic sites are located in the C-terminal regions of the collagen P4H $\alpha$ subunits and contain four conserved catalytically critical residues, two histidines and one aspartate that bind the Fe$^{2+}$ atom and a lysine that binds the C-5 carboxyl group of 2-oxoglutarate (23). The peptide-substrate-binding domain of the collagen P4Hs is separate from the catalytic domain and is located between residues 138 and 244 in the 517-residue human $\alpha$(I) subunit (24).

To study the proposed processive reaction mechanism further, we determined the $K_m$ values of the PHY-1/PHY-2/(PDI-2)$_2$ tetramer and the PHY-1/human PDI dimer for peptide substrates of different lengths, the PHY-1/human PDI dimer being chosen because it is formed much more effectively than any PHY/PDI-2 dimer. Lower $K_m$ values with increasing substrate chain length were observed in the case of both the enzyme tetramer and the dimer, the results suggesting that these occur on account of the higher affinity of the peptide-substrate-binding domain for long peptides rather than through processive binding. To study the separate roles of the two catalytic sites in one collagen P4H tetramer, we generated mutant recombinant C. elegans PHY-1/PHY-2/(PDI-2)$_2$ tetramers in which the catalytic site of one $\alpha$ subunit was inactivated by mutation of either the Fe$^{2+}$-binding aspartate or the 2-oxoglutarate-binding lysine residue. The P4H activities of the mutant tetramers were markedly less than 50% of that of the wild-type, indicating that the remaining single wild-type catalytic site in the mutants does not function entirely independently.
MATERIALS AND METHODS

Expression and Analysis of the Recombinant PHY-1/PHY-2/(PDI-2)$_2$ Tetramer and the PHY-1/Human PDI Dimer in Insect Cells – High Five insect cells (Invitrogen) were cultured on plates in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (Euroclone). The cells, seeded at a density of $0.6 \times 10^6$/ml, were coinfected at a multiplicity of 5 with viruses coding for the *C. elegans* PHY-1 (15), PHY-2 (20) and the *C. elegans* PDI-2 polypeptides (25), or the *C. elegans* PHY-1 and human PDI polypeptides (26). The cells were harvested 72 h after infection, washed with a solution of 0.15M NaCl and 0.02M phosphate, pH 7.4, homogenized in a 0.1M NaCl, 0.1 M glycine, 0.1% Triton X-100, 10 µM dithiothreitol and 0.01 M Tris buffer, pH 7.8, and centrifuged at 10,000 x g for 20 min. Samples of the Triton X-100 soluble supernatants were analyzed by 8% nondenaturing PAGE followed by Coomassie Blue staining.

Production of Recombinant Non-hydroxylated Collagen Polypeptides in the Yeast *Pichia pastoris* - To generate a *Pichia* strain expressing recombinant nonhydroxylated type I collagen $\alpha$1 chain fragments of 510 and 100 amino acids, cDNA products originating from the codons for amino acids 683 and 1093 of the pro$\alpha$1(I) chain, respectively, and extending to the codon for the last amino acid of the collagenous domain were generated by PCR. These fragments had an artificial *Eco*RI site at their 5’ ends and a stop codon followed by a *Not*I site at their 3’ ends. After digestion with the corresponding restriction enzymes, the cDNA fragments were ligated to an *Eco*RI-*Not*I-digested pPIC9K expression vector (Invitrogen) in frame with the yeast $\alpha$-mating factor secretory signal. The constructs were linearized with *Sal*I and electroporated into a GS115 *Pichia* strain (Invitrogen) according to the manufacturer’s instructions (27). The strains were cultured in 100-ml shaker flasks in a buffered glycerol complex medium, pH 6.0, with 1 g/l yeast extract and 2 g/l peptone. Expression was induced in a buffered minimal methanol medium, pH 6.0, and methanol was added every 12 h to a final concentration of 0.5%. The culture medium was collected 60 h after induction, concentrated in an Ultrafree 0.5 centrifugal filter unit with a Biomax-5 membrane (Millipore) and the recombinant type I collagen polypeptide fragments were analyzed by 12% SDS-PAGE followed by Coomassie Blue staining.
Site-directed Mutagenesis and Expression of Mutant C. elegans PHY-1/PHY-2/(PDI-2) Tetramers in Insect Cells – The C. elegans PHY-1 aspartate 407 (codon GAT) and lysine 486 (codon AAA) residues were converted individually to asparagine (codon AAT) and alanine (codon GCA), respectively, and the PHY-2 aspartate 405 (codon GAC) and lysine 484 (codon AAA) residues to asparagine (codon AAC) and alanine (codon GCA), respectively. The mutagenesis steps were performed in a pVL1392 vector (Invitrogen) containing the full-length C. elegans phy-1 (15) and phy-2 (20) cDNAs using the QuikChange™ XL Site-Directed Mutagenesis Kit (Stratagene). The recombinant baculovirus constructs were cotransfected into Spodoptera frugiperda Sf9 cells with a modified Autographa californica nuclear polyhedrosis virus DNA using the BaculoGold transfection kit (PharMingen). The resultant virus pools were collected and amplified (28). Sf9 or H5 Insect cells were infected with the individual mutant viruses, harvested, and homogenized as described above. The remaining cell pellets were solubilized in 1% SDS and expression of the mutant polypeptides was analyzed by 8% SDS-PAGE followed by Coomassie Blue staining. To express mutant C. elegans P4H tetromers, the cells were coinfected with the viruses coding for the mutant PHY-1 and wild-type PHY-2, or wild-type PHY-1 and mutant PHY-2 polypeptides together with the virus coding for the C. elegans PDI-2. The cells were homogenized as described above and samples of the Triton X-100 soluble fractions were analyzed by 8% nondenaturing PAGE followed by Western blotting with a polyclonal PHY-1 antibody (20) and ECL immunodetection (Amersham Biosciences). The expression levels of the wild-type and mutant tetraters were compared by densitometry of the ECL films using a GS-710 calibrated imaging densitometer (Bio-Rad).

P4H Activity Assay - The P4H activity of the recombinant wild-type and mutant C. elegans tetraters and the PHY-1/human PDI dimer was analyzed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate (29) using the peptides (Pro-Pro-Gly)₃ and (Pro-Pro-Gly)₁₀ (Peptide Institute) and the recombinant nonhydroxylated 100 and 510-amino acid type I collagen α1 chain fragments as substrates. The amounts of the wild-type and mutant enzyme tetraters were adjusted to be equal in the activity assay reactions on the basis of densitometry of the tetramer bands on the ECL Western blots of
nondenaturing PAGE gels. In the case of the mutant tetramers produced with the wild-type PHY-1 and mutant PHY-2 subunits, the amount of activity generated by the PHY-1/PDI-2 dimer was subtracted from the activity values. The activity of the PHY-1/PDI-2 dimer was determined based on the amount generated in insect cells expressing only the PHY-1/PDI-2 dimer and densitometry comparisons of the amount of this dimer and the dimer formed in addition to the mutant tetramer. Since assembly of the recombinant PHY-2/PDI-2 dimers in insect cells is very inefficient, lying below the detection limit of Western blot analysis (20), the activity generated by the recombinant PHY-2/PDI-2 dimer was regarded as nonsignificant and was not subtracted. $K_m$ values for the peptide substrates of different lengths and for the cosubstrates Fe$^{2+}$ and 2-oxoglutarate were determined as described previously (29). In some experiments the amount of 4-hydroxyproline formed was determined by a colorimetric method in samples hydrolyzed with 6 M HCl at 120°C overnight (30).

RESULTS

*Increasing Substrate Chain Length Reduces the $K_m$ Values of the PHY-1/PHY-2/(PDI-2)$_2$ Tetramer and PHY-1/Human PDI Dimer Equally* - According to the hypothesis of processive action of the two peptide-binding sites in the collagen P4H tetramer, an enzyme with two such sites should be much more efficient in hydroxylating long peptide substrates than an enzyme with only one site, whereas the synergistic relation between the two sites should not be evident in the hydroxylation of short peptides (21, 22).

To study the suggested co-operation between the two peptide-binding sites in more detail, a recombinant *C. elegans* PHY-1/PHY-2/(PDI-2)$_2$ tetramer, i.e. a P4H with two peptide-binding and catalytic sites, and the PHY-1/human PDI dimer, i.e. a P4H with one peptide-binding and one catalytic site, were produced in insect cells (Fig. 1) and their $K_m$ values for peptide substrates of four lengths determined using an activity assay based on the hydroxylating-coupled decarboxylating of 2-oxo[1-$^{14}$C]glutarate. It has previously been reported that the $K_m$ for a nonhydroxylated full-length proα1 chain of type I procollagen, with a collagenous domain of about 1000 amino acids, is increased 5-10-fold when one of the two
peptide-binding sites in the collagen P4H tetramer is blocked by a photoaffinity label, while this effect was not seen with a short 15-amino-acid peptide (Pro-Pro-Gly)$_5$ (21). The substrates used here were the synthetic peptides (Pro-Pro-Gly)$_5$, and (Pro-Pro-Gly)$_{10}$ and recombinant nonhydroxylated 100 and 510-amino-acid fragments of the α1 chain of human type I collagen produced in the yeast P. pastoris (Fig. 2). The two recombinant fragments have 11 and 55 potential hydroxylation sites, respectively, calculated from their amino acid sequence, while (Pro-Pro-Gly)$_5$ and (Pro-Pro-Gly)$_{10}$ have five and ten such sites. All the $K_m$ values of the PHY-1/human PDI dimer were found to be about 1.5-2.5 times those of the PHY-1/PHY-2/(PDI-2)$_2$ tetramer (Table I), but the chain length of the peptide substrate was found to have an identical effect on both enzyme forms, as their $K_m$ values for the 510-amino-acid fragment were 0.03% of those for (Pro-Pro-Gly)$_5$, and 0.8-1.4% of those for (Pro-Pro-Gly)$_{10}$ (Fig. 3 and Table I). The effect of increasing peptide chain length on the $K_m$ values was also seen when expressed in terms of the molar concentration of the hydroxylatable -X-Pro-Gly- triplets (Table I). Interestingly, the $K_m$ values of the enzyme tetramer and dimer for the 100-amino-acid fragment, when expressed per peptide, were 24 and 28% of those for (Pro-Pro-Gly)$_{10}$, respectively, although these two peptides differ by only one in terms of the number of possible hydroxylation sites. The $K_m$ values for this collagen fragment thus clearly corresponded to the number of all -X-Y-Gly- triplets in it rather than the number of -X-Pro-Gly- triplets (Table I).

Expression Levels of Mutant C. elegans PHY Polypeptides in Insect Cells Are Comparable to Those of the Wild-Type Polypeptides - The effect of inactivation of one of the two catalytic sites in the C. elegans PHY-1/PHY-2/(PDI-2)$_2$ tetramer on the enzyme activity was studied by generating mutant recombinant baculoviruses which were used to infect insect cells. Baculoviruses coding for C. elegans PHY polypeptides in which the iron-binding aspartate residue has been replaced by an asparagine, PHY-1(Asp$^{407}$→Asn) or PHY-2(Asp$^{405}$→Asn), or the lysine binding the C-5 carboxyl group of the 2-oxoglutarate has been replaced by an alanine, PHY-1(Lys$^{486}$→Ala) or PHY-2(Lys$^{484}$→Ala), were generated and used to infect Sf9 or H5 cells. The cells were harvested 72 h after infection, homogenized in a buffer containing 0.1% Triton X-100, and centrifuged. The cell pellets were further
solubilized in 1% SDS, and the Triton X-100 and SDS-soluble proteins were analyzed by SDS-PAGE under reducing conditions. In agreement with the data previously reported for the C. elegans PHY-1 and PHY-2 polypeptides (15, 20), the mutant PHY-1 and PHY-2 polypeptides formed insoluble aggregates and their efficient extraction required the use of 1% SDS (Fig. 4). The expression levels of the mutant PHY-1 and PHY-2 polypeptides were found to be comparable to those of the wild-type polypeptides (Fig. 4).

Inactivation of One Catalytic Site in the Collagen P4H Tetramer Inactivates the Enzyme by More than Half - To express mutant C. elegans collagen P4H tetramers in which binding of the iron atom to one catalytic site was prevented by a point mutation, insect cells were coinfected with viruses coding for PHY-1(Asp<sup>407</sup>→Asn) and PHY-2 or PHY-1 and PHY-2(Asp<sup>405</sup>→Asn) together with the virus coding for the C. elegans PDI-2 (Fig. 5). Correspondingly, to express tetramers in which binding of the 2-oxoglutarate to one catalytic site is abolished, insect cells were coinfected with viruses coding for PHY-1(Lys<sup>486</sup>→Ala) and PHY-2 or PHY-1 and PHY-2(Lys<sup>484</sup>→Ala), and PDI-2 (Fig. 5). The cells were harvested 72 h after infection, homogenized in a Triton X-100 buffer, and the soluble fractions analyzed by nondenaturing PAGE followed by Western blotting with a PHY-1 antibody (Fig. 6). All the mutant PHY polypeptides became assembled into mixed tetramers with the wild-type polypeptides, a small amount of wild-type or mutant PHY-1/PDI-2 dimer also being detected (Fig. 6).

The P4H activity of the mutant enzyme tetramers generated in the insect cell homogenates was analyzed using (Pro-Pro-Gly)<sub>10</sub> as a substrate (Table II). The amounts of wild-type and mutant enzyme tetramers in the activity assay reactions were adjusted to be equal on the basis of densitometry of the tetramer bands on the ECL Western blots of non-denaturating PAGE gels (Fig. 6). Furthermore, the activity generated by the wild-type PHY-1/PDI-2 dimer (Fig. 6, lanes 1, 3 and 5), 4-6% (data not shown), was subtracted from the activity values as described in Materials and Methods. Inactivation of the iron-binding aspartate in either the PHY-1 or the PHY-2 polypeptide led to a marked reduction in enzyme activity, the values being 5-8% of that of the wild-type tetramer (Table II). Inactivation of the lysine that binds the C-5 carboxyl group of the 2-oxoglutarate in either of the PHY
polypeptides likewise caused a distinct decrease in the activity, which was 25-27% of that of the wild-type tetramer (Table II). The activities of all the mutant tetramers were thus reduced by more than 50%, inactivation of one of the iron-binding sites having a more severe effect, however, than inactivation of one of the 2-oxoglutarate-binding sites, the former resulting in about a 3-5-fold lower residual activity (Table II). The catalytic sites in the PHY-1 and PHY-2 polypeptides seem to be equally important, as no difference was observed between the activity values obtained with the enzymes having a mutation in either the PHY-1 or PHY-2 polypeptide (Table II). None of the mutations affected the peptide substrate binding of the enzymes, as the $K_m$ values for (Pro-Pro-Gly)$_{10}$ were, within the accuracy of the assays, identical between the wild-type and mutant enzymes (Table II). Mutation of the iron-binding or 2-oxoglutarate-binding site in one of the two catalytic subunits had no effect on the binding of these cosubstrates to the remaining wild-type subunit, either, as the $K_m$ values of the mutant enzymes for these cosubstrates were identical to those of the wild-type enzyme (Table III).

The P4H activity of the mutant enzyme tetramers was also determined with saturating concentrations of the recombinant 100 and 510-amino-acid nonhydroxylated α1(I) collagen fragments as substrates (Table IV). The results were very similar to those obtained with (Pro-Pro-Gly)$_{10}$ above. Mutation of the iron-binding aspartate or the 2-oxoglutarate-binding lysine in either of the PHY polypeptides reduced the enzyme activity to 5-8% and 18-33% of that of the wild-type enzyme, respectively (Table IV).

The Less Severe Effect of Inactivation of One of the Two 2-Oxoglutarate-binding Sites Is Not Due to an Increased Uncoupled 2-Oxoglutarate Decarboxylation - A surprising finding was that mutation of an iron-binding residue in one of the two catalytic sites had a 3-5-fold more severe effect on the enzyme activity than the abolition of 2-oxoglutarate-binding to one of the catalytic subunits. One possible explanation for this difference is that binding of the peptide substrate to the mutant catalytic site enhanced the rate of an uncoupled decarboxylation of 2-oxoglutarate at the wild-type site, i.e. decarboxylation without subsequent hydroxylation of the peptide substrate (1-3). Collagen P4Hs are known to catalyze uncoupled decarboxylation at a low rate even in the presence of saturating
concentrations of their peptide substrates, and binding of peptides that do not act as substrates, such as poly(L-proline) or (Gly-Pro-Ala)$_n$, is known to increase the rate of uncoupled decarboxylation (1-3). In such a case the hydroxylation of peptide substrates by the PHY-1/PHY-2/(PDI-2)$_2$ tetramer, containing one mutant 2-oxoglutarate-binding site, might in fact be decreased much more than the value measured in the 2-oxoglutarate decarboxylation assay. This possibility was studied by analyzing the amounts of 4-hydroxyproline formed during the reaction by a colorimetric method and comparing these values (Table V) to those measured with the 2-oxoglutarate decarboxylation assay (Table II). Essentially identical values were obtained in both assays indicating that inactivation of one of the two 2-oxoglutarate-binding sites in a tetramer indeed had a less severe effect on the rate of the hydroxylation reaction than mutation of one of the two iron-binding sites.

**DISCUSSION**

Vertebrate collagen P4Hs are tetrameric enzymes containing two catalytic α subunits (1-3). It has previously been shown that the chain length of the peptide substrate has a major effect on the $K_m$ values of the vertebrate enzymes, which decrease markedly with increasing chain length, while the maximal reaction velocity is not affected (1-3). This phenomenon has been explained by a processive mechanism of binding of the two peptide-substrate-binding sites of the collagen P4H tetramers, leading to much faster binding by overcoming the diffusional constraints on the rate of association between the enzyme and the individual hydroxylatable sites (21, 22). Here we studied the $K_m$ values of a *C. elegans* PHY-1/PHY-2/(PDI-2)$_2$ tetramer and a PHY-1/human PDI dimer for substrates of varying lengths. Our data clearly demonstrate that the effect of increasing peptide chain length on the $K_m$ values is identical with both enzyme forms and thus does not require the presence of two peptide-substrate-binding sites and two catalytic sites. Nevertheless, the $K_m$ values of the *C. elegans* PHY-1/human PDI dimer for all peptide substrates were about 1.5-2.5 times those of the *C. elegans* PHY-1/PHY-2/(PDI-2)$_2$ tetramer (Table I), possibly due to small conformational differences between the enzyme tetramer and dimer.
The peptide-substrate-binding domain of human type I collagen P4H has recently been located between residues 138-244 in the α(I) subunit and shown to be separate from the catalytic C-terminal region (24). Determination of the $K_d$ values of the recombinant human type I and type II collagen P4H peptide-substrate-binding domains for the binding of synthetic peptides varying in lengths from 2 to 10 -Pro-Pro-Gly- triplets showed that the length dependence is observed even with the individual domains, the $K_d$ values measured with the domains being very similar to the $K_m$ values for the enzyme tetramers (31). The results obtained here provide further evidence for the hypothesis that the peptide length dependence most probably results from a more effective binding of longer peptides to the peptide-substrate-binding domain rather than from the processive action of two binding sites.

The $K_m$ values of the collagen P4H dimer and tetramer for the 100-amino-acid type I collagen α1 chain fragment were 24-28% of those for (Pro-Pro-Gly)$_{10}$, although the fragment has only one additional possible hydroxylation site relative to the synthetic peptide (Table I). The interaction of peptide substrates with collagen P4Hs has been shown to be affected by the amino acid present in the X position of the -X-Pro-Gly- triplets to be hydroxylated, proline in the X position probably giving the highest maximal reaction velocity, and by amino acids in other parts of the peptide (2). The present data clearly indicate that interaction of the 100-residue collagen fragment with the P4H tetramer and dimer was influenced by all its -X-Y-Gly- triplets rather than only the -X-Pro-Gly- triplets.

Surprisingly, mutation of the iron-binding aspartate or the lysine that binds the C-5 carboxyl group of the 2-oxoglutarate in either of the PHY polypeptides was found to inactivate the *C. elegans* PHY-1/PHY-2/(PDI-2)$_2$ tetramer by more than half, indicating that the remaining wild-type catalytic site is not capable of functioning entirely independently. This cannot be due to impaired binding of Fe$^{2+}$ or 2-oxoglutarate to the remaining wild-type catalytic site, as the $K_m$ values of the mutant enzymes for these cosubstrates were identical to those of the wild-type enzyme. In the first half-reaction of the P4H catalytic cycle, binding of 2-oxoglutarate and an oxygen molecule to the iron atom bound to three catalytic residues [His$^{412}$, Asp$^{414}$ and His$^{483}$ in the case of the human α(I) subunit] leads to the formation of a ferryl ion, which during the second half-reaction hydroxylates the C-4 of a substrate proline.
residue pointing towards it (1-3, 23). Binding studies with the peptides (Gly-Pro-4Hyp)$_3$ and (Gly-Pro-Pro)$_3$ have shown that the $K_d$ of the peptide-substrate-binding domain of the human type I collagen P4H for the hydroxylated peptide is more than one order of magnitude higher than that for the nonhydroxylated peptide, so that the peptide-substrate-binding domain most probably contributes to the release of the hydroxylated product (31). When the hydroxylation reaction in one of the two catalytic sites of a collagen P4H tetramer is inhibited by a mutation, it is highly likely that the long peptide substrate will remain bound at the mutant subunit, thus interfering with the “free search” performed by the functional catalytic subunit and the peptide substrate for new hydroxylation events.

The two catalytic sites in the *C. elegans* PHY-1/PHY-2/(PDI-2)$_2$ tetramer seem to have equally important roles in the hydroxylation of a peptide substrate, as essentially identical activity values were obtained with enzymes having a catalytic site mutation in either the PHY-1 or PHY-2 polypeptide. Although the activity of the mutant enzymes was reduced by more than half with respect to that of the wild-type enzyme, our results clearly show that the two catalytic sites are both functional in the enzyme tetramer. An unexpected finding was, however, that inactivation of one of the two iron-binding sites had a more severe effect on the enzyme activity than that of one of the two 2-oxoglutarate-binding sites. Mutation of either the iron-binding aspartate or the positively charged residue that binds the C-5 carboxyl group of the 2-oxoglutarate at both catalytic sites of the human type I collagen P4H tetramer and the lysyl hydroxylase homodimer and at the single catalytic site of the monomeric *A. thaliana* P4H has been shown to inactivate these enzymes completely (23, 32-34), and the present data indicate a complete inactivation of the PHY-1/PDI dimer by these mutations (Table II). A possible explanation for the marked difference between the two types of mutant tetramer studied here is that binding of the peptide substrate to the mutant subunit caused a conformational change that was more severe in the case of the mutants involving one of the two iron-binding sites than those involving one of the 2-oxoglutarate-binding sites.

The present data show that efficient hydroxylation of long peptide substrates by collagen P4Hs does not require the presence of two catalytic sites and two peptide-binding sites, and provide further support for the importance of the peptide-substrate-binding domain as the
main determinant for the properties of the binding of various peptides to collagen P4Hs. The results also show that although inactivation of one of the two catalytic sites in a collagen P4H tetramer does not affect the $K_m$ values of reaction cosubstrates or the peptide substrate, the activity of the enzyme is reduced by more than half. Slow release of the nonhydroxylated peptide from the mutant subunit most probably impairs the rapid search performed by the functional subunit for a new -X-Pro-Gly- sequence in the substrate to enable a productive hydroxylation event, and this is likely to explain the unexpectedly large decrease in the reaction rate when one of the catalytic sites is inactivated.

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FOOTNOTES

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¶To whom correspondence should be addressed. Tel: +358-8-537 5740; Fax: +358-8-537 5811; Email: johanna.myllyharju@oulu.fi.

¹The abbreviations used are: ER, endoplasmic reticulum; P4H, prolyl 4-hydroxylase; PDI, protein disulfide isomerase
FIG. 1. Nondenaturing PAGE analysis of the expression of the *C. elegans* PHY-1/PHY-2/(PDI-2)_{2} collagen P4H tetramer and the PHY-1/human PDI dimer in insect cells. Triton-X-100 soluble fractions of lysates from cells expressing the *C. elegans* PHY-1/PHY-2/(PDI-2)_{2} collagen P4H tetramer (*lane 1*) or the PHY-1/human PDI dimer (*lane 2*) were analyzed by 8% nondenaturing PAGE followed by Coomassie Blue staining. The enzyme tetramer and dimer are indicated by *T* and *D*, respectively.

FIG. 2. SDS-PAGE analysis of recombinant non-hydroxylated type I collagen α1 chain fragments expressed in the yeast *Pichia pastoris*. Medium samples from yeast strains expressing the 100 and 510-amino-acid fragments of the α1(I) chain (*lanes 1 and 2*), respectively, were concentrated and analyzed by 12% SDS-PAGE under reducing conditions followed by Coomassie Blue staining.

FIG. 3. Effect of increasing peptide length on the *K_{m}* values of the *C. elegans* PHY-1/PHY-2/(PDI-2)_{2} tetramer and the PHY-1/human PDI dimer. The lengths of the peptide substrates in amino acids are indicated on the X-axis and the *K_{m}* values of the *C. elegans* PHY-1/PHY-2/(PDI-2)_{2} tetramer (*filled squares*) and the PHY-1/human PDI dimer (*open squares*) on a logarithmic scale on the Y-axis.

FIG. 4. SDS-PAGE analysis of the expression of recombinant wild-type and mutant *C. elegans* PHY-1 and PHY-2 polypeptides. SDS-soluble fractions of lysates from insect cells expressing PHY-1 (*lane 1*), PHY-2 (*lane 2*), PHY-1(Lys^{486}→Ala) (*lane 3*), PHY-2(Lys^{484}→Ala) (*lane 4*), PHY-1(Asp^{407}→Asn) (*lane 5*) and PHY-2(Asp^{405}→Asn) (*lane 6*) polypeptides were analyzed by 8% SDS-PAGE under reducing conditions followed by Coomassie Blue staining. The *arrow* indicates the position of the PHY-1 and PHY-2 polypeptides.
FIG. 5. **Schematic representation of the wild-type and mutant C. elegans PHY-1/PHY-2/(PDI-2)\textsubscript{2} tetramers.** The PHY-1 and the PHY-2 subunits are indicated by horizontal and vertical hatching, respectively, while the PDI-2 subunits are indicated in white. The mutations preventing binding of the iron or 2-oxoglutarate cofactor are indicated.

FIG. 6. **Nondenaturing PAGE analysis of the expression of recombinant wild-type and mutant C. elegans PHY-1/PHY-2/(PDI-2)\textsubscript{2} tetramers and the PHY-1/PDI-2 dimer.** Triton X-100-soluble fractions of lysates from insect cells expressing C. elegans PHY-1/PHY-2/(PDI-2)\textsubscript{2} (lane 1), PHY-1(Lys\textsuperscript{486}→Ala)/PHY-2/(PDI-2)\textsubscript{2} (lane 2), PHY-1/PHY-2(Lys\textsuperscript{484}→Ala)/(PDI-2)\textsubscript{2} (lane 3), PHY-1(Asp\textsuperscript{407}→Asn)/PHY-2/(PDI-2)\textsubscript{2} (lane 4), and PHY-1/PHY-2(Asp\textsuperscript{405}→Asn)/(PDI-2)\textsubscript{2} (lane 5) enzyme tetramers and the PHY-1/PDI-2 dimer (lane 6) were analyzed by 8% nondenaturing PAGE followed by Western blotting using an anti-PHY-1 antibody. The enzyme tetramer and dimer are indicated by $T$ and $D$, respectively.
TABLE I

*K*m values of the C. elegans PHY-1/PHY-2/(PDI-2)2 collagen P4H tetramer and the PHY-1/human PDI dimer for peptide substrates of varying lengths

*K*m values were determined using soluble extracts of insect cells expressing the recombinant collagen P4Hs as sources of the enzymes.

| Substrate | PHY-1/PHY-2/(PDI-2)2 | PHY-1/human PDI |
|-----------|----------------------|-----------------|
|           | *K*m of peptide      | *K*m of -X-Pro-Gly- | *K*m of peptide | *K*m of -X-Pro-Gly- |
| (Pro-Pro-Gly)s | 660 3300 | 1490 | 7450 |
| (Pro-Pro-Gly)10 | 25 250 | 36 | 360 |
| 100-amino-acid α1(I) collagen fragment | 6 66 | 10 | 110 |
| 510-amino-acid α1(I) collagen fragment | 0.2 11 | 0.5 | 27.5 |

μM
**TABLE II**

P4H activity of the wild-type and mutant PHY-1/PHY-2/(PDI-2) enzymes with (Pro-Pro-Gly)$_{10}$ as a substrate

| Enzyme                                   | P4H activity$^b$ | $K_m$ for (Pro-Pro-Gly)$_{10}$ |
|------------------------------------------|------------------|---------------------------------|
|                                           | dpm/50µl         | % of the wild-type              | µM    |
| PHY-1/PHY-2/(PDI-2)$_2$                  | 20350 ± 3240     | 100$^c$                         | 25    |
| PHY-1(Asp$^{407}$→Asn)/PHY-2/(PDI-2)$_2$| 1020 ± 610       | 5 ± 4                           | 26    |
| PHY-1/PHY-2(Asp$^{405}$→Asn)/(PDI-2)$_2$| 1630 ± 410       | 8 ± 2$^c$                      | 40    |
| PHY-1(Lys$^{486}$→Ala)/PHY-2/(PDI-2)$_2$| 5090 ± 1020      | 25 ± 5                         | 20    |
| PHY-1/PHY-2(Lys$^{484}$→Ala)/(PDI-2)$_2$| 5500 ± 410       | 27 ± 2$^c$                      | 30    |
| PHY-1/human PDI                          | 17840 ± 5040     | 100$^d$                         | 36    |
| PHY-1(Asp$^{407}$→Asn)/human PDI        | 0                | 0$^d$                          |       |
| PHY-1(Lys$^{486}$→Ala)/human PDI        | 0                | 0$^d$                          |       |

$^a$Equal amounts of the wild-type and mutant enzymes were used, based on densitometry of the tetramer bands in nondenaturing PAGE analysis.

$^b$The activity assay was performed with 120 µM (Pro-Pro-Gly)$_{10}$ as a substrate. The values are given as mean ± S.D. for 3 independent experiments.

$^c$Activity generated by the PHY-1/PDI-2 dimer was subtracted from these values.

$^d$The activity values of the mutant PHY-1/human PDI dimers were compared with that of the wild-type dimer.
TABLE III

*Kₘ* values of the wild-type and mutant *C. elegans* mixed tetramers for *Fe²⁺* and 2-oxoglutarate

| Enzyme                              | *Kₘ* | Fe²⁺ | 2-Oxoglutarate |
|-------------------------------------|------|------|----------------|
|                                     | µM   |      |                |
| PHY-1/PHY-2/(PDI-2)                | 1    | 80   |
| PHY-1(Asp⁴⁰⁷⇒Asn)/PHY-2/(PDI-2)    | 1    |      | N.D.           |
| PHY-1/PHY-2(Asp⁴⁰⁵⇒Asn)/(PDI-2)    | 1    |      | N.D.           |
| PHY-1(Lys⁴⁸⁶⇒Ala)/PHY-2/(PDI-2)    | N.D. | 80   |
| PHY-1/PHY-2(Lys⁴⁸⁴⇒Ala)/(PDI-2)    | N.D. | 80   |
TABLE IV

*P4H activity of the wild-type and mutant PHY-1/PHY-2/(PDI-2)*2 tetramers with peptide substrates of varying lengths*

| Enzyme                                               | P4H activity | 100-amino-acid α1(I) collagen fragment | 510-amino-acid α1(I) collagen fragment | % of wild-type |
|------------------------------------------------------|--------------|----------------------------------------|----------------------------------------|----------------|
| PHY-1/PHY-2/(PDI-2)*2                                |              | 100                                    | 100                                    |                |
| PHY-1(Asp*407→Asn)/PHY-2/(PDI-2)*2                   | 6            | 5                                      |                                        |                |
| PHY-1/PHY-2(Asp*405→Asn)/(PDI-2)*2                   | 8c           | 6                                      |                                        |                |
| PHY-1(Lys*486→Ala)/PHY-2/(PDI-2)*2                    | 18           | 22                                     |                                        |                |
| PHY-1/PHY-2(Lys*484→Ala)/(PDI-2)*2                    | 23c          | 33c                                    |                                        |                |

*a*Equal amounts of the wild-type and mutant enzymes were used, based on densitometry of the tetramers bands in nondenaturing PAGE analysis.

*b*The activity assays were performed in the presence of saturating concentrations of the substrates. Similar values were obtained in 3 independent experiments.

*c*Activity generated by the PHY-1/PDI-2 dimer was subtracted from these values.
**TABLE V**

*Formation of 4-hydroxyproline by the wild-type and mutant PHY-1/PHY-2/(PDI-2) enzymes with (Pro-Pro-Gly)$_{10}$ as a substrate*

| Enzyme                                              | 4-Hydroxyproline formed$^b$ | µg    | % of the wild-type |
|-----------------------------------------------------|-----------------------------|-------|--------------------|
| PHY-1/PHY-2/(PDI-2)$_2$                             |                             | 6.20  | 100$^c$            |
| PHY-1(Asp$^{407} \rightarrow$Asn)/PHY-2/(PDI-2)$_2$|                             | 0.30  | 5                  |
| PHY-1/PHY-2(Asp$^{405} \rightarrow$Asn)/(PDI-2)$_2$|                             | 0.50  | 8$^c$              |
| PHY-1(Lys$^{486} \rightarrow$Ala)/PHY-2/(PDI-2)$_2$|                             | 1.70  | 27                 |
| PHY-1/PHY-2(Lys$^{484} \rightarrow$Ala)/(PDI-2)$_2$|                             | 1.75  | 28$^c$             |

$^a$Equal amounts of the wild-type and mutant enzymes were used, based on densitometry of the tetramer bands in nondenaturing PAGE analysis.

$^b$The formation of 4-hydroxyproline was analyzed with 160 µM (Pro-Pro-Gly)$_{10}$ as a substrate. Similar values were obtained in 3 independent experiments.

$^c$The amount of 4-hydroxyproline generated by the PHY-1/PDI-2 dimer was subtracted from these values.
Fig. 1 Kukkola et al.

Fig. 2 Kukkola et al.
Fig. 3 Kukkola et al.

Fig. 4 Kukkola et al.
Fig. 5 Kukkola et al.

Fig. 6 Kukkola et al.
Collagen Prolyl 4-hydroxylase tetramers and dimers show identical decreases in Km values for peptide substrates with increasing chain length. Mutation of one of the two catalytic sites in the tetramer inactivates the enzyme by more than half.

Liisa Kukkola, Peppi Koivunen, Outi Pakkanen, Antony P. Page and Johanna Myllyharju

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