Characterization of Recombinant Human Prolyl 3-Hydroxylase Isoenzyme 2, an Enzyme Modifying the Basement Membrane Collagen IV*

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The single 3-hydroxyproline residue in the collagen I polypeptides is essential for proper fibril formation and bone development as its deficiency leads to recessive osteogenesis imperfecta. The vertebrate prolyl 3-hydroxylase (P3H) family consists of three members, P3H1 being responsible for the hydroxylation of collagen I. We expressed human P3H2 as an active recombinant protein in insect cells. Most of the recombinant polypeptide was insoluble, but small amounts were also present in the soluble fraction. P3H1 forms a complex with the cartilage-associated protein (CRTAP) that is required for prolyl 3-hydroxylation of fibrillar collagens. However, coexpression with CRTAP did not enhance the solubility or activity of the recombinant P3H2. A novel assay for P3H activity was developed based on that used for collagen prolyl 4-hydroxylases (C-P4Hs) and lysyl hydroxylases (LHs). A large amount of P3H activity was found in the P3H2 samples with (Gly-Pro-4Hyp)₁₃ as a substrate. The Kᵣ and Kᵢ values of P3H2 for 2-oxoglutarate and its certain analogues resembled those of the LHs rather than the C-P4Hs. Unlike P3H1, P3H2 was strongly expressed in tissues rich in basement membranes, such as the kidney. P3H2 hydroxylated more effectively two synthetic peptides corresponding to sequences that are hydroxylated in collagen IV than a peptide corresponding to the 3-hydroxylation site in collagen I. These findings suggest that P3H2 is responsible for the hydroxylation of collagen IV, which has the highest 3-hydroxyproline content of all collagens. It is thus possible that P3H2 mutations may lead to a disease with changes in basement membranes.

Collagen synthesis involves many unusual co-translational and post-translational modifications, including the formation of 4-hydroxyproline (4Hyp), 3-hydroxyproline, and hydroxylysine in -X-Pro-Gly-, -Pro-4Hyp-Gly-, and -X-Lys-Gly-sequences, respectively. These reactions take place within the lumen of the endoplasmic reticulum and are catalyzed by collagen prolyl 4-hydroxylases (C-P4Hs), prolyl 3-hydroxylases (P3Hs), and lysyl hydroxylases (LHs), which belong to the 2-oxoglutarate-dependent dioxygenases (1–4). 4-Hydroxyproline residues have an essential role in the folding of the newly synthesized collagen polypeptide chains into thermally stable triple-helical molecules and their amounts in different collagen types are relatively similar, around 100 residues per 1000 amino acids. Hydroxylysine residues have two important functions: they are essential for the stability of the intermolecular cross-links that provide the collagen fibrils with their tensile strength and mechanical stability and they serve as attachment sites for carbohydrate units (1). The functions of 3-hydroxyproline residues are not known in detail, but their presence in the X positions of repeating -X-4Hyp-Gly- triplets has a destabilizing effect on the triple helix (5, 6), despite a standard superhelix symmetry and lack of large structural alterations (7). The amount of 3-hydroxyproline varies markedly between different collagen types, from 1 residue per 1000 amino acids in collagen I to 10–15 in collagen IV (8, 9). It has been suggested that 3-hydroxyproline residues may generate specific regions of lower stability within the triple helix that may be necessary for the assembly of certain supramolecular structures, e.g. the meshwork structure formed by collagen IV in basement membranes (6).

Although P3H was partially purified and characterized from chick embryos more than 25 years ago (10), it was cloned from chicken tissues only recently (11). Data base analyses of vertebrate genomes pointed to the existence of a family with three members, named P3Hs 1–3 (11). P3H1 was found to be identical to leprecan, or growth suppressor 1 (Gros1), which had previously been identified as a basement membrane-associated glycoprotein in rats and as a potential growth suppressor in mice (12, 13). The leprecan-like 1 protein, which corresponds to P3H2, has been shown to be localized to the endoplasmic reticulum and Golgi network and to be expressed in many secretory tissues (14). The lengths of the processed human P3H 1, 2, and 3 polypeptides are 736, 708, and 736 amino acids, respectively, each having a C-terminal endoplasmic reticulum retention signal, and the sequence identity is 46% between P3Hs 1 and 2 and 38% between P3Hs 2 and 3 (11). The catalytically critical residues identified in 2-oxoglutarate-dependent dioxygenases (2, 15, 16) are conserved in the P3Hs, but otherwise no significant amino acid sequence similarity is observed.
between the P3Hs, C-P4Hs, and LHs. Chick P3H1 copurifies with cyclophilin B and the cartilage-associated protein (CRTAP), indicating that it forms a tight complex with these endoplasmic reticulum-resident proteins (11). It localizes specifically to tissues that express fibril-forming collagens (11).

Most cases of osteogenesis imperfecta (OI) are caused by dominant mutations in the two genes that code for the polypeptide chains of collagen I (17). Interestingly, mice lacking CRTAP suffer from a severe OI-like syndrome with short stature, kyphosis, and severe osteoporosis (18). Biochemical analyses showed that the single 3-hydroxyproline residue present in the polypeptide chains of collagens I and II is absent in the mutant mice and that collagen fibrillogenesis is altered, indicating that this unique modification has an essential role in bone formation (18). Furthermore, these results indicate that although P3H1 can hydroxylate collagen chains in vivo without the presence of CRTAP (11), the latter is required for its efficient function in vivo. In humans, a deficiency in CRTAP and a concomitant lack of or reduction in prolyl 3-hydroxylation was found to lead to recessive OI, ranging in severity from neonatal lethality to a milder phenotype depending on the nature of the mutation (18, 19). Similarly, mutations in the gene coding for human P3H1 have recently been shown to cause recessive lethal or severe OI (20).

We report here on the expression of human P3H2 as an active recombinant protein in insect cells. Most of the recombinant P3H2 polypeptide was found in the insoluble fraction, but small amounts were also present in the soluble fraction. Coexpression with CRTAP did not improve the solubility of the recombinant P3H2 polypeptide and had no effect on its P3H activity level. P3H2 efficiently hydroxylated a synthetic (Gly-Pro-4Hyp)$_n$ peptide, its $K_m$ for this peptide being slightly lower than that of C-P4H-I for a peptide of a corresponding length. Small but distinct differences in $K_m$ values were found between P3H2 and C-P4H-I for reaction cosubstrates Fe$_2$O$_4$ and small amounts were also present in the soluble fraction. Sequence analyses showed that the single 3-hydroxyproline residue present in the polypeptide chains of collagens I and II is absent in the mutant mice and that collagen fibrillogenesis is altered, indicating that this unique modification has an essential role in bone formation (18). Furthermore, these results indicate that although P3H1 can hydroxylate collagen chains in vivo without the presence of CRTAP (11), the latter is required for its efficient function in vivo. In humans, a deficiency in CRTAP and a concomitant lack of or reduction in prolyl 3-hydroxylation was found to lead to recessive OI, ranging in severity from neonatal lethality to a milder phenotype depending on the nature of the mutation (18, 19). Similarly, mutations in the gene coding for human P3H1 have recently been shown to cause recessive lethal or severe OI (20).

**EXPERIMENTAL PROCEDURES**

**PCR Analysis of P3H mRNA Expression in Various Mouse and Human Tissues**—Expression of mouse and human P3H1–3 mRNAs in various tissues was studied by PCR analysis of the mouse and human multiple tissue cDNA (MTC) panel I and the human fetal MTC panel (Clontech) according to the manufacturer’s protocol, using the Phusion polymerase (Finnzymes). The primer pairs were 5’-CCCTTTGTTGGAT-CGGACGTATGCGG-3’ and 5’-ATAGAGGGGGCCTGGTCTC-AGCCGG-3’ for mouse P3H1, 5’-CTCACAGCTTCTTCA-TGGCCACACCCC-3’ and 5’-AAATCTCCGGCCGCTCA-ATGG-3’ for mouse P3H2, 5’-GGACACTGGATACGGTTCGCG-3’ and 5’-CAGCTGCAGAGACCCACAC-3’ for mouse P3H3, 5’-GGATCCGGATCTGAGACTCC-3’ and 5’-GACCCGGTGGACCCATCC-3’ for human P3H2, 5’-TGTGGATGACTGAGTCTCG-3’ and 5’-GGACGTTATCCAGGAACC-3’ for human P3H2, and 5’-TCAGTGTTGGAATCTCCGCT-3’ and 5’-CAGCTGCTGGAAAT-CCTCGG-3’ for human P3H3. Glyceraldehyde 3-phosphate dehydrogenase primers (Clontech) were used to analyze the amounts of templates present in the MTC panels. The PCR products were analyzed on a 1% agarose gel and their identities were verified by sequencing on an automated DNA sequencer (ABI Prism 377, Applied Biosystems).

**Prolyl 3-Hydroxylase Isoenzymes and CRTAP in Insect Cells**—The human P3H1 cDNA was amplified by PCR from fetal Marathon-Ready cDNA and lung QUICK-Clone cDNA (BD Biosciences) in two fragments. The PCR primers 5’-GCGGGCCGCCGGTGCTCGCGGCCTCGTGGAG-3’ and 5’-CAAAGGGATACTACAAATCCCATCAGAGCC-3’ were used to obtain the 5’-end and primers 5’-TTTTTGGAATTTACCTTTGATGCCG-3’ and 5’-CGGCTGTTATCCTCTTCTGATCATTGGAATCCC-3’ for the 3’-end. The primers had an internal P3H1 EcoRI restriction site (bold) and artificial NotI and XbaI (underlined) restriction sites to facilitate cloning into NotI-Xbal-digested pVL1392 (Pharmingen). The full-length human P3H2 cDNA was amplified by PCR from a human kidney cDNA pool (Clontech) using primers 5’-GGAGAATCTGGATCAACCCGTTTGCAGCCTCC-3’ and 5’-GCTGCTGATGAGATAATATCCATTCCATC-3’. Artificial BglII and XbaI restriction sites (underlined) were included in the primers to facilitate cloning into similarly digested pVL1392. The P3H3 cDNA was amplified by PCR from Ad5 and ovarian carcinoma cell lines (human cell line MTC panel, Clontech) using primers 5’-GGAGAATCTGGATCAACCCGTTTGCAGCCTCC-3’ and 5’-GCTGCTGATGAGATAATATCCATTCCATC-3’. Artificial BglII and XbaI restriction sites (underlined) were included in the primers to facilitate cloning into similarly digested pVL1392. The sequences were verified on an ABI Prism 377 automated DNA sequencer (Applied Biosystems).

The recombinant vectors were cotransfected into Spodoptera frugiperda (Sf9) insect cells with modified Autographa californica nuclear polyhedrosis virus DNA (BaculoGold, Pharmingen) by calcium phosphate transfection, and the recombinant viruses were amplified (21). Monolayer cultures of Sf9 cells in TNM-FH medium (Sigma) supplemented with 10% fetal bovine serum (HyClone) were infected with the recombinant viruses at a multiplicity of 5 and a cell density of 5 × 10$^6$ per 100-mm plate. The cells were harvested 72 h after infection,
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washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4 (PBS), and homogenized in a solution of 0.1 M NaCl, 0.1 M glycine, 2 mM CaCl₂, 10 μM dithiothreitol, 0.1% Triton X-100, and 0.01 M Tris, pH 7.8, including the complete EDTA-free protease inhibitor mixture (Roche Diagnostics), and centrifuged at 13,000 × g for 20 min. The remaining pellets were solubilized in 1% SDS, and aliquots of both fractions were analyzed by 8% SDS-PAGE under reducing conditions followed by Coomassie Blue staining or ECL Western blotting (Amersham Biosciences) with polyclonal antibodies against P3H1 and P3H2 (see below) at 1:5000 and 1:500 dilutions, respectively. N-terminal sequencing of the putative recombinant P3H polypeptides present in the 0.1% Triton X-100 and 1% SDS-soluble fractions was performed using samples transferred to a ProBlott™ membrane (Applied Biosystems) and a Procise™ 492 sequencer (Applied Biosystems).

Analysis of P3H Activity—P3H activity was analyzed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-¹⁴C]glutarate (22). The reaction was performed in a final volume of 0.5 ml, which contained 50–75 μl of the Triton X-100 extracts of the insect cells as a source of the enzyme, differing amounts of a peptide substrate (Gly-Pro-4Hypro)₅ (Innovagen), 0.025 μmol of FeSO₄, 0.05 μmol of 2-oxo-[1-¹⁴C]glutarate, 1 μmol of ascorbate, 30 μg of catalase (Sigma), 0.05 μmol of dithiothreitol, 1 mg of bovine serum albumin, and 25 μmol of Tris-HCl buffer, adjusted to pH 7.8 at 25 °C. The enzyme reaction was carried out at 37 °C for 30 min. In addition to experiments the above substrate peptide was replaced by the synthetic peptides Ser-Lys-Gly-Glu-Gln-Gly-Phe-Met-Gly-Pro-4Hypro-Gly-Pro-Gln-Gly-Gln-4Hypro-Gly-Leu-4Hypro-Gly or Pro-Thr-Gly-Pro-Arg-Gly-Phe-Pro-Gly-Pro-4Hypro-Gly-Pro-Asp-Gly-Ley-4Hypro-Gly-Ser-Met-Gly corresponding to two known prolyl 3-hydroxylation sites in the collagen IV α1 chain (23), respectively, or Leu-Asn-Gly-Leu-4Hypro-Gly-Pro-Gly-Pro-4Hypro-Gly-Pro-Arg-Gly-Phe-Pro-Gly-Pro-4Hypro-Gly-Pro-Asp-Gly-Ley-4Hypro-Gly-Ser-Met-Gly corresponding to two known prolyl 3-hydroxylation sites in the collagen I α1 chain at position 986 of the triple helix (18). Triton X-100 extracts of insect cells expressing human C-P4H-I (24) and a peptide substrate (Pro-Pro-Gly)₅ were used in control experiments.

Production of P3H Antibodies, Immunohistochemistry, and Immunoelectron Microscopy—Polyclonal rabbit antibodies were produced against mouse P3H1 and P3H2 using synthetic peptides PEEVIPKRLQEOKQSE and MGKKPSPPKIGRDLEG corresponding to residues 398–413 and 422–437 of mouse P3H1 and P3H2, respectively (Innovagen). Both antibodies were purified on a Hi-Trap H HP column (Amersham Biosciences). The adult mouse specimens for use in the immunofluorescence studies were immediately frozen in liquid nitrogen and stored at −70 °C. Samples from these were cut into 5-μm cryosections on SuperFrost plus glass (Menzel-Gläser) slides and the sections were fixed in pre-cooled acetone for 10 min at 4 °C. After rinsing with PBS, pH 7.2, nonspecific antibody binding was blocked by incubating the sections with 5% goat serum in PBS, pH 7.2, for 12 h at 22 °C. Incubation with the primary antibodies was performed at 22 °C for 90 to 150 min using 1:100 and 1:200 dilutions of the P3H1 and P3H2 antibodies, respectively, and a 1:300 dilution of a polyclonal collagen IV antibody (Chemicon). The slides were washed in PBS, pH 7.2, 5 times for 5 min, followed by a 30-min incubation in a 1:300 dilution of a goat anti-rabbit Alexa Fluor 568 secondary antibody (Invitrogen). After washing 7 times for 5 min in PBS, pH 7.2, the slides were mounted with Immumount (Thermo Electron Corporation). Control sections were stained with the secondary antibody alone. Preimmune sera from the rabbits immunized with the P3H1 and P3H2 peptides were also used as negative controls. The samples were examined under an Olympus BX50 microscope (Olympus) and photographed with a DP50 digital camera (Olympus).

Fresh adult mouse kidney was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer with 2.5% sucrose, pH 7.4, for 2 h. Small pieces of tissue were immersed in 2.3 M sucrose and frozen in liquid nitrogen. Thin cryosections were cut with a Leica Ultracut UCT™ microtome. The sections for immunolabeling were first incubated in 0.05 M glycine in PBS followed by incubation in 5% bovine serum albumin with 0.1% cold water fish skin gelatin (Aurion) in PBS. The antibodies and gold conjugate were diluted in 0.1% bovine serum albumin/cold water fish skin gelatin (Aurion) in PBS. All washings were performed in 0.1% bovine serum albumin/cold water fish skin gelatin in PBS. The sections were then incubated with an antibody to P3H2 (dilution 1:300) for 60 min followed by protein A-gold complex (size 10 nm) for 30 min. The controls were prepared by carrying out the labeling procedure without any primary antibody. The sections were embedded in methylcellulose and examined in a Philips CM100 transmission electron microscope (FEI Company). Images were captured by CCD camera equipped with a TCL-EM-Menu, version 3, from Tietz Video and Image Processing Systems GmbH (Gaunting).

RESULTS

Expression of the P3H 1–3 mRNAs in Various Mouse and Human Tissues—We used PCR analysis of multitissue cDNA panels to study the expression of the P3H isoenzyme mRNAs in various mouse and human tissues. P3H2 mRNA was expressed in all the tissues studied except for adult human brain and skeletal muscle (Fig. 1). The highest expression levels in mouse tissues were seen in the lung, skeletal muscle, and kidney, in adult human tissues in the placenta, lung, liver, and kidney, and in fetal human tissues in the heart, spleen, lung, liver, skeletal muscle, and kidney (Fig. 1). P3H1 mRNA was expressed in all the tissues except for mouse brain and spleen (Fig. 1). The highest levels in mouse tissues were seen in the liver and skeletal muscle, in adult human tissues in the placenta, lung, liver, kidney, and pancreas and in fetal human tissues in the spleen, lung, liver, skeletal muscle, and kidney (Fig. 1). In addition to the expected 344-bp human P3H1 PCR product, a longer product was also amplified with the P3H1 oligonucleotides (Fig. 1B), which proved to contain the intron 7 sequence. This variant corresponds to the previously identified Gros1-S variant that contains part of the intron 5 sequence and the entire intron 7 sequence, leading to a premature stop codon and the generation of a truncated 363-amino acid polypeptide (13) instead of the 736-residue full-length polypeptide. This truncated polypeptide cannot be an active P3H, as it lacks the catalytically critical residues identified in the 2-oxoglutarate dioxygenases.
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Expression of an Active Recombinant Human P3H2 in Insect Cells—Recombinant human P3H1, P3H2, and P3H3 polypeptides were expressed in Sf9 insect cells. The cells were harvested 72 h after infection, homogenized in a buffer containing 0.1% Triton X-100, and centrifuged, and the remaining pellets were further solubilized in 1% SDS. The samples were analyzed by SDS-PAGE under reducing conditions followed by Coomassie Blue staining or Western blotting with polyclonal antibodies raised against synthetic mouse P3H1 and P3H2 peptides. Most of the recombinant P3H1 and P3H2 polypeptides were found in the SDS-soluble fraction (Fig. 2), but a significant amount of P3H1 and a small amount of P3H2 were also seen in the Triton X-100-soluble fraction (Fig. 2). The Coomassie Blue staining and comparison with samples from non-infected Sf9 cells suggested that the P3H3 polypeptide was present only in the SDS fraction (Fig. 2A). Western blot analysis showed that the P3H1 and P3H2 antibodies were specific for their target isoenzymes (Fig. 2, B and C), except that the P3H2 antibody additionally recognized a nonspecific insect cell protein with a lower molecular weight, which was also stained in the noninfected control cells (Fig. 2B). Furthermore, the same insect cell protein was stained with preimmune serum collected from the rabbits immunized with the P3H2 peptide (data not shown).

The expression of the P3H polypeptides was further verified by N-terminal sequencing. The prediction for the P3H1 signal peptide cleavage site is uncertain, but it is located between residues 18 and 23, whereas the sizes of the signal peptides of P3H2 and P3H3 are predicted to be 24 and 20 amino acids, respectively. N-terminal sequencing indicated that P3H1 and P3H2 polypeptides with N termini of ASQAEVESEA and GPPDSPRREL, respectively, were present in both the Triton X-100 and SDS fractions, whereas a P3H3 sequence EPPGLTQLSP was present only in the SDS fraction. These results agree with those obtained in the SDS-PAGE and Western blot analyses above.

To study whether the recombinant P3H polypeptides had P3H activity, Triton X-100-soluble fractions of the cell homogenates were assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate (22). A large amount of P3H activity was obtained in the P3H2 sample when the synthetic peptide (Gly-Pro-4Hyp)₃ was used as the substrate.

### Table 1

| Enzyme | Peptide concentration µM | Activity M dpm/75 µl |
|--------|--------------------------|---------------------|
| P3H2   | 25                       | 5,200               |
|        | 50                       | 9,500               |
|        | 100                      | 16,300              |
|        | 300                      | 24,200              |
| P3H1   | 100                      | <200                |
|        | 300                      | <200                |
| C-P4H-I-ⁿ | 100  | <200                |

ⁿ The Triton X-100-soluble fraction of insect cells expressing C-P4H-I was used as a negative control.

**FIGURE 1.** Expression of the P3H isoenzyme mRNAs in mouse and human tissues. Mouse (A) and human (B) multitissue cDNA panels were analyzed by PCR with primers specific for P3H2, P3H1, and P3H3. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH) primers were used to confirm that equal amounts of the cDNA templates were present in the samples.

**FIGURE 2.** Analysis of the expression of recombinant human P3H1, P3H2, and P3H3 polypeptides in insect cells. SDS-PAGE analysis under reducing conditions followed by Coomassie Blue staining (A) and Western blotting with a P3H2 (B) or P3H1 (C) antibody of Triton X-100-soluble (odd numbered lanes) and SDS-soluble (even numbered lanes) proteins from Sf9 insect cells infected with a virus encoding P3H1 (lanes 1 and 2), P3H2 (lanes 3 and 4), or P3H3 (lanes 5 and 6), and non-infected Sf9 cells (lanes 7 and 8). Lanes 7 and 8 in B were grouped from a different part of the same gel as lanes 1–6 in B.
substrate (results of a typical experiment are shown in Table 1), although the amount of P3H2 in the Triton X-100-soluble fraction was relatively low (Fig. 2). Variation of the concentration of the peptide substrate (Fig. 3A) or any of the cosubstrates (as shown for 2-oxoglutarate in Fig. 3B) gave typical Michaelis-Menten kinetics.

In contrast, despite the higher amount of P3H1 in the Triton X-100 fraction, no P3H activity was obtained in the P3H1 sample (Table 1). This might be due to the presence of six single nucleotide deviations in the cloned P3H1 cDNA, which led to amino acid changes as compared with the GenBank sequence. We therefore generated several versions of the P3H1 cDNA by site-directed mutagenesis to change the cDNA obtained to be entirely or partly identical with the GenBank P3H1 sequence, but none of the coded recombinant P3H1 polypeptides had P3H activity. No P3H activity was obtained in the P3H3 sample, either, which agrees with this polypeptide being insoluble in the buffer containing Triton X-100 (Fig. 2). As CRTAP copurifies with the chick P3H1 (11) and is required for prolyl 3-hydroxylation in vivo (18, 19), we coexpressed CRTAP with the P3H polypeptides in insect cells, but this did not lead to any increased solubility or activity of the recombinant P3Hs (data not shown).

Catalytic Properties of Recombinant Human P3H2—Because the P4Hs and P3Hs require Fe^{2+}, 2-oxoglutarate, O_2, and ascorbate (1, 2, 4) the _K_m_ values of P3H2 for the substrate (Gly-Pro-4Hyp)_5 and the cosubstrates Fe^{2+}, 2-oxoglutarate, and ascorbate and the _K_i_ values for certain efficient C-P4H inhibitors were determined (Table 2). The _K_m_ for (Gly-Pro-4Hyp)_5 was 70 _M_ being 2–5-fold lower than that of C-P4H-I for a corresponding (Pro-Pro-Gly)_5 peptide (Table 2). In control experiments, P3H2 did not hydroxylate the C-P4H substrate (Pro-Pro-Gly)_5 and purified C-P4H-I did not hydroxylate the (Gly-Pro-4Hyp)_5 peptide (Table 2). The _K_m_ of P3H2 for Fe^{2+}, 0.5 _M_, was one-fourth of that of C-P4H-I, whereas the _K_m_ for 2-oxoglutarate, 80 _M_, was 4 times higher (Table 2). The 2-oxoglutarate analogue pyridine 2,4-dicarboxylate, an effective competitive inhibitor of C-P4Hs, was also an efficient competitive inhibitor of P3H2, whereas pyridine 2,5-dicarboxylate inhibited P3H2 only at very high concentrations (Table 2). P3H2 was inhibited only very inefficiently by poly(l-proline), an effective C-P4H-I inhibitor (Table 2).

Expression of the P3H2 Polypeptide in Various Mouse Tissues—3-Hydroxyproline is found most abundantly in collagen IV, which is one of the major components of basement membranes (8, 9). Immunohistochemical analysis has shown that the expression of P3H1 in 16-day-old chick embryos is localized to tissues that produce fibrillar collagens, such as the dermis, tendon, cartilage, large blood vessels, and connective tissue septae, whereas only restricted expression of P3H1 or no detectable expression was seen in tissues where basement membrane collagens prevail (11). We therefore used the P3H1 and P3H2 antibodies generated here to study the expression of these isoenzymes in adult mouse tissues. In general, P3H2 was found to be expressed strongly in tissues where basement membrane collagens, and thus collagen IV, are abundant, whereas staining for P3H1 was only weak in these tissues (Fig. 4).

Strong staining for P3H2 was seen in the kidney tubular cells, whereas weak staining, if any, was seen in the glomerular cells (Fig. 4). The staining pattern for P3H2 in the kidney resembled that of collagen IV with the exception that collagen IV was also strongly expressed in the glomerulus and the surrounding Bow-
Recombinant Human P3H2 Hydroxylates Collagen IV Sequences More Efficiently Than a Collagen I Sequence—The immunohistochemical data showed that P3H2 is expressed in tissues rich in basement membranes and it is thus likely to be involved in the hydroxylation of collagen IV. To study this, synthetic peptides corresponding to two known prolyl 3-hydroxylation sites in the α1 chain of collagen IV (23) as well as a peptide corresponding to the only hydroxylation site in the α1 chain of collagen I (18) were tested as substrates for the recombinant P3H2, and the $K_m$ and $V_{max}$ values were determined (Table 3). The $V_{max}$ values are given as relative values to that obtained with the (Pro-4Hyp-Gly)$_5$ peptide in the same experiment (Table 3). All three peptides were hydroxylated by P3H2. The $K_m$ values of P3H2 for the collagen IV peptide Ser-Lys-Gly-Glu-Gln-Gly-Phe-Met-Gly-Pro-4Hyp-Gly-Pro-Gln-Gly-Gln-4Hyp-Gly-Leu-4Hyp-Gly and the collagen I peptide Leu-Asn-Gly-Leu-4Hyp-Gly-Pro-Ile-Gly-Pro-4Hyp-Gly-Pro-Arg-Gly-Arg-Thr-Gly-Asp-Ala-Gly were identical, about 260 μM, but the $V_{max}$ obtained with the collagen IV peptide was about 1.5-fold higher than that obtained with the collagen I peptide (Table 3). The $K_m$ and $V_{max}$ values were determined (Table 3). Based on the kinetic data both collagen IV peptides were thus hydroxylated by P3H2 more efficiently than the collagen I peptide. It is thus evident that the amino acid sequence surrounding the core hydroxylation site -Pro-4Hyp-Gly- has a marked effect on the hydroxylation efficiency of P3H2.

DISCUSSION

The P3H partially purified and characterized from a chick embryo extract and from rat kidney cortex about 30 years ago...
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(10, 25) was in all probability isoenzyme 1, as its purification procedure included affinity chromatography on denatured rat skin collagen. Its \(K_m\) values were determined for the reaction cosubstrates using a biologically prepared proline-labeled non-hydroxylated procollagen substrate, which had been first fully hydroxylated in the Y position prolines of its -X-Y-Gly- triplets hydroxylated in the Y position prolines of its -X-Y-Gly- triplets with a purified C-P4H in vitro (10). The \(K_m\) values for \(\text{Fe}^{2+}\), 2-oxoglutarate, \(\text{O}_2\), and ascorbate were found to be essentially identical to those of C-P4H-I (10). Determination of exact \(K_m\) and \(K_i\) values is rather difficult with this assay, however, as its linear range is narrow. We show here for the first time that the activity of P3H can be conveniently analyzed by a similar assay that is routinely used to study P4H and LH activity (22, 26), i.e. by determination of the amount of hydroxylation-coupled decarboxylation of 2-oxo-[1-14C]glutarate when a short synthetic peptide (Gly-Pro-4Hyp), is used as a substrate. Our results show that certain differences existed between the catalytic properties of P3H2 and C-P4H-I. The \(K_m\) of P3H2 for 2-oxoglutarate was four times higher than that of the three C-P4H isoenzymes, which all have identical \(K_m\) values for 2-oxoglutarate, and about the same as those of the three LH isoenzymes (4, 15, 27). Interestingly, the 2-oxoglutarate analogue pyridine 2,5-dicarboxylate, which is an effective competitive inhibitor of the C-P4Hs, was a very poor inhibitor of P3H2, a property in which it resembled the LHs (4, 9). The 2-oxoglutarate binding site of the 2-oxoglutarate dioxygenases consists of two major subsites (1, 2, 4). Subsite I is a positively charged residue in positions +9 or 10 with respect to the second \(\text{Fe}^{2+}\)-binding histidine that binds the C5 carboxyl group of the 2-oxoglutarate, whereas subsite II consists of two coordination sites of the enzyme-bound \(\text{Fe}^{2+}\) and is chelated by the C1–C2 moiety. The P3Hs and LHs resemble each other in that the basic residue binding the C5 carboxyl group of 2-oxoglutarate in all their isoenzymes is an arginine, whereas it is a lysine in all the C-P4Hs (11, 15, 28). Based on this difference and the kinetic data, the structures of the 2-oxoglutarate-binding sites of the P3Hs and LHs appear to share common features that are different from those of the C-P4Hs.

The chick P3H1 was cloned based on N-terminal sequencing of proteins that became bound to gelatin Sepharose, i.e. an affinity substrate prepared from denatured fibrillar collagen (11). Although leprecan, which is now known to be P3H1, was originally identified as a basement membrane-associated proteoglycan (12), the chick P3H1 was found to be present in tissues that express fibrillar collagens and not in tissues rich in basement membranes (11). It was therefore suggested that P3H1 is likely to modify fibrillar collagens (11), and this suggestion has recently been verified by the lack of the single 3-hydroxyproline residue in collagen I polypeptides of patients with mutations in the gene for P3H1 (20). We have shown here that P3H2 expression localizes to tissues rich in basement membranes and the recombinant P3H2 efficiently hydroxylates synthetic peptides corresponding to known hydroxylation sites in the \(\alpha1\) chain of collagen IV. This isoenzyme is thus likely to be responsible for the prolyl 3-hydroxylation of collagen IV. The 3-hydroxyproline content of collagen IV is much higher than that of fibrillar collagens, but the function of prolyl 3-hydroxylation in the case of collagen IV is unknown. As the lack of the single 3-hydroxyproline in collagen I polypeptides normally generated by P3H1 was found to interfere with the proper assembly and secretion of this collagen, leading to severe OI, it will be of interest to study the synthesis and assembly of collagen IV molecules in P3H2 null mice. The recombinant P3H2 also hydroxylated a synthetic peptide corresponding to the only hydroxylation site in the \(\alpha1\) chain of collagen I, but less efficiently than the collagen IV peptides. Based on the tissue localization of P3H2 and the fact that mutations of human P3H1 cause a recessive lethal or severe OI (20), P3H2 is not likely to play a significant role in the hydroxylation of fibrillar collagens.

The six collagen IV chains assemble into three kinds of collagen IV molecules, with compositions \(\alpha1(IV)_2\alpha2(IV)_1\) and relative mass percentages of 30, 40, and 30, respectively. The \(\alpha1(IV)_1\) chain is typically hydroxylated to the extent of 70% to 100%, whereas the \(\alpha2(IV)_1\) chain is more variable, with values ranging from 15 to 40%. The \(\alpha1(IV)_2\) chain is hydroxylated to a lesser extent, typically between 15 and 30%.

**TABLE 3**

| Peptide                        | \(K_m\) \(\mu M\) | \(V_{\text{max}}\) % | \|              |
|-------------------------------|-------------------|---------------------|----------------|
| Ser-Lys-Gly-Glu-Gln-Gly-Phe-Met-Gly-Pro-4Hyp-Gly-Pro-Gln-Gly-Gln-4Hyp-Gly-Leu-4Hyp-Gly\(^{a}\) | 260 ± 70          | 150 ± 40            |                |
| Pro-Thr-Gly-Pro-Arg-Gly-Phe-Pro-Gly-Pro-4Hyp-Gly-Pro-Asp-Gly-Lys-4Hyp-Gly-Ser-Met-Gly\(^{a}\) | 70 ± 10           | 150 ± 30            |                |
| Leu-Asp-Gly-Leu-4Hyp-Gly-Pro-Ile-Gly-Pro-4Hyp-Gly-Pro-Arg-Gly-Pro-Arg-Gly-Trp-Gly-Asp-Ala-Gly\(^{a}\) | 260 ± 70          | 100 ± 30            |                |

\(^{a}\) The \(V_{\text{max}}\), values are expressed relative to that obtained with the [Pro-4Hyp-Gly]$_2$ peptide.

\(^{b}\) Peptide corresponding to a known prolyl 3-hydroxylation site in the \(\alpha1\) chain of collagen IV.

\(^{c}\) Peptide corresponding to the only prolyl 3-hydroxylation site in the \(\alpha1\) chain of collagen I.
α3(IV)α4(IV)α5(IV), and α5(IV),α6(IV) (29). Molecular recognition sequences in the non-collagenous NC1 domains of the chains govern the selection of partner chains for both the assembly of triple helical collagen IV molecules and their subsequent assembly into networks. Collagen IV networks composed of α1(IV) and α2(IV) chains are found in all basement membranes, but the network consisting of the α3(IV), α4(IV), and α5(IV) chains predominates in the glomerular and tubular basement membranes of the kidney in humans and rodents (29–31). P3H2 was strongly expressed in the tubular cells of the mouse kidney, suggesting that it has an important role in the formation of both types of collagen IV network in the kidney. In view of the tissue expression of P3H2, it is conceivable that human P3H2 mutations may lead to a disease phenotype characterized by changes in basement membranes.

The recombinant P3H polypeptides, especially P3H2 and P3H3, were poorly soluble in insect cells. Chick P3H1 has been shown to copurify in a tight complex with CRTAP and cyclophilin B (11). Although CRTAP and cyclophilin B were not found to affect the in vitro activity of purified chick P3H1 (11), CRTAP has been shown to be important for efficient prolyl 3-hydroxylation in vivo (18, 19). However, because coexpression of CRTAP with the recombinant human P3H polypeptides in insect cells did not enhance either their solubility or their activity, it seems that CRTAP may not be required for the maintenance of P3H polypeptides in a catalytically active soluble conformation, but rather its presence in the complex may ensure efficient interaction with the nascent procollagen chains in vivo and be necessary for their hydroxylation and proper folding. Although CRTAP mRNA is expressed in most tissues (32), its highest expression levels are seen in the skeletal muscle; Instruction Manual, Pharmingen, San Diego, CA.

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