Prolyl 3-Hydroxylase 1, Enzyme Characterization and Identification of a Novel Family of Enzymes*

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The collagen prolyl hydroxylases are enzymes that are required for proper collagen biosynthesis, folding, and assembly. They reside within the endoplasmic reticulum and belong to the group of 2-oxoglutarate and iron-dependent dioxygenases. Although prolyl 4-hydroxylase has been characterized as an αβ2 tetramer in which protein disulfide isomerase is the β subunit with two different α subunit isoforms, little is known about the enzyme prolyl 3-hydroxylase (P3H). It was initially characterized and shown to have an enzymatic activity distinct from that of prolyl 4-hydroxylase, but no amino acid sequences or genes were ever reported for the mammalian enzyme. Here we report the characterization of a novel prolyl 3-hydroxylase enzyme isolated from embryonic chicks. The primary structure of the enzyme, which we now call P3H1, demonstrates that P3H1 is a member of a family of prolyl 3-hydroxylases, which share the conserved residues present in the active site of prolyl 4-hydroxylase and lysyl hydroxylase. P3H1 is the chick homologue of mammalian leprecan or growth suppressor 1. Two other P3H family members are the genes previously called MLAT4 and GRCB. In this study we demonstrate prolyl 3-hydroxylase activity of the purified enzyme P3H1 on a full-length procollagen substrate. We also show it to specifically interact with denatured collagen and to exist in a tight complex with other endoplasmic reticulum-resident proteins. Immunohistochemistry with a monoclonal antibody specific for chick P3H1 localizes P3H1 specifically to tissues that express fibrillar collagens, suggesting that other P3H family members may be responsible for modifying basement membrane collagens.

The biosynthesis of collagen involves a number of unique post-translational modifications that are catalyzed by several specific enzymes. Hydroxylation of the appropriate prolyl and lysyl residues to 4-hydroxyprolyl, 3-hydroxyprolyl, and hydroxylysyl residues is a modification required inside the cell to ensure proper folding and assembly of procollagen. These modifications occur because of the enzymatic activities of specific rough endoplasmic reticulum-resident (rER) proteins (1, 2). Prolyl 4-hydroxylase (P4H), prolyl 3-hydroxylase (P3H), and lysyl hydroxylase (LH) all belong to the group of 2-oxoglutarate dioxygenases and require Fe2+, 2-oxoglutarate, O2, and ascorbate for their activity. The 2-oxoglutarate is stoichiometrically decarboxylated during hydroxylation, with one atom of the O2 molecule incorporated into succinate, whereas the other is incorporated into the hydroxy group formed on the proline or lysine residue.

P4Hs catalyze the formation of 4-hydroxyproline by the hydroxylation of prolines in Xaa-Pro-Gly- sequences in collagens and more than 15 other proteins that have collagen-like domains (1, 2). P4H exists as an αβ2 tetramer with a molecular mass of ~240,000 daltons. Two isoforms of the catalytic α subunit have been characterized and shown to form [α(II)3β3] and [α(II)3β2] tetramers, the β subunit of which is identical to the chaperone protein disulfide isomerase (PDI) (3, 4). A third isoform of the α subunit, cloned just recently, was characterized and found to be expressed at lower levels than the other two isoforms in human tissues (5). There also exists a family of cytoplasmic prolyl 4-hydroxylases, the hypoxia-inducible factor P4Hs that catalyze the hydroxylation of the α subunit of hypoxia-inducible transcription factor and play a key role in the response of cells to hypoxia (6, 7). These enzymes hydroxylate Leu-Xaa-Xaa-Leu-Ala-Pro-Tyr- and -Leu-Xaa-Xaa-Leu-Ala-Pro-Ala-sequences (8, 9).

The lysyl hydroxylases are a family of three proteins, LH1–3, that catalyze the hydroxylation of -Xaa-Lys-Gly-sequences. Their activity is essential for the stability of the intermolecular collagen cross-links, and their hydroxy groups serve as attachment sites for carbohydrate units (1). Although there is little primary sequence identity between the P4Hs and LysHs, they share conserved catalytic residues in their active sites (2, 10).

In collagen, 4-hydroxylation of proline residues is crucial for the formation of the triple helix (11). Almost all proline residues in the Yaa position of the -Gly-Xaa-Yaa- repeated sequence are hydroxylated to 4R-hydroxyproline by prolyl 4-hydroxylase. 3(S)-hydroxyproline is found in almost all collagens in the sequence -Gly-3Hyp-3Hyp-Gly- (12–14). The role of 3(S)-hydroxyproline in the Xaa position of all collagens is not well understood. The extent of 3-hydroxylation varies with the different types of collagens and occurs in the largest amounts in collagen types IV and V (15, 16). Prolyl 3-hydroxylase and prolyl 4-hydroxylase activities have been shown to be derived from separate proteins (17, 18), however no amino acid sequences or genes have ever been reported for prolyl 3-hydrox-

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‡ The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY463528, AY463529, and AY463530.

¶ The abbreviations used are: rER, rough endoplasmic reticulum; P3H, prolyl 3-hydroxylase; P4H, prolyl 4-hydroxylase; LH, lysyl hydroxylase; PDI, protein disulfide isomerase; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; CYPB, cyclophilin B; CRTAP, cartilage-associated protein.
Prolyl 3-Hydroxylase 1

Prolyl 3-Hydroyxlase 1 was reported to be purified 5000-fold from a chick embryo extract, and its molecular mass was estimated to be 160,000 daltons by gel filtration (19). In this study we report the identification and characterization of a prolyl 3-hydroxylase purified from chick embryos. The translated sequence of the cloned prolyl 3-hydroxylase gene, as well as partial amino acid sequences from the purified protein, indicates that it is the chick homologue of lepricon or growth suppressor 1 (Gros1). Lepricon was originally identified from a rat cDNA library as a basement membrane-associated proteoglycan, which was also shown to possess the endoplasmic reticulum retention signal KDE1 (20). Subsequently the mouse homologue to lepricon, Gros1, was cloned and found to be a potential growth suppressor on chromosome 1 (21). Lepricon was then classified as a member of the 2-oxoglutarate- and iron-dependent dioxygenases using sequence profile searches and protein fold recognition to propose a biological function for unknown proteins (22). Based on the results presented here, we choose to call the protein prolyl 3-hydroxylase 1. We also have identified two homologous gene sequences that we predict to be other members of this novel P3H1 family. We show this enzyme to have prolyl 3-hydroxylase activity in a previously described assay with full-length procollagen (26). Gelatin-Sepharose affinity chromatography, used previously to identify proteins that bind to denatured collagen (23, 24), was used here to demonstrate the ability of P3H1 to specifically bind to denatured collagen as well as to interact with other ER proteins as a complex. Finally, immunohistochemistry using a monoclonal antibody to P3H1 demonstrates its presence in tissues that express fibrillar collagens.

EXPERIMENTAL PROCEDURES

Gelatin-Sepharose Affinity Chromatography and Enzyme Purification—P3H and P4H were isolated from 15-day-old chick embryos by affinity chromatography on gelatin-Sepharose (Amersham Biosciences) (23, 24) with the following modifications. 12 dozen chicken embryos were mixed with an equal volume of 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and proteinase inhibitors (5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, 1 mM mg/ml pepstatin A, and 1 mg/ml leupeptin). Homogenization was carried out in a Waring blender at maximum speed for 3 min. This and all subsequent cheesecloth and Miracloth, and run over a gelatin-Sepharose 4B column in the same protease inhibitors as described above, treated with 1 ml of 1 M KH2PO4, pH 5.0. The column was washed with at least two bed volumes of buffer A (20). The reaction was performed in a final volume of 1.0 ml, which contained 0.05 M NaCl, and 0.1% Triton X-100 and then dialyzed into PBS (Invitrogen), 42 C/ml of purified chick prolyl 4-hydroxylase for 4 h at 37 C in a final volume of 10 ml containing 0.08 mM FeSO4, 2 mM ascorbic acid, 0.5 mM 2-oxoglutarate, and 0.05 M Tris-HCl buffer, pH 7.8. The reaction was stopped by adding 1 ml of 1 M KH2PO4, pH 5.0. The reaction was then performed at 50 °C for 10 min immediately before use.

The prolyl 3-hydroxylase reactions were performed as described previously (17, 25, 26) but with the following modifications. The enzyme reaction was carried out for 60 min at 24 °C in a final volume of 2.0 ml containing 1 × 106 dpm of L-[2,3-3H]proline-labeled substrate, 0.08 mM FeSO4, 2 mM ascorbic acid, 0.5 mM 2-oxoglutarate, and 2 mg/ml bovine serum albumin, 0.1 mM dithiothreitol, and 0.05 M NaCl. The reaction was then performed at 50 °C for 4 h at 37 C in a final volume of 10 ml containing 0.08 mM FeSO4, 2 mM ascorbic acid, 0.5 mM 2-oxoglutarate, and 0.05 M Tris-HCl, pH 7.8. The reaction was stopped by adding 0.5 ml of 10 ml of trichloroacetic acid, and the tritiated water formed was assayed by vacuum distillation of the whole reaction mixture (25, 26). A 1.8-ml aliquot of the tritiated water was mixed with 10 ml of Ecolimulin liquid scintillation mixture (ICN) and counted in a Beckman LS8000TD liquid scintillation counter.

The prolyl 4-hydroxylase activity was assayed by a method based on the hydroxylation-coupled decarboxylation of 2-oxo-L-[1-14C]glutarate (25). The reaction was performed in a final volume of 1.0 ml, which contained 0.1 mg of (Pro-Pro-Gly)9-H2O as substrate, 2 mM ascorbic acid, 0.05 mM FeSO4, 0.1 mM dithiothreitol, 2 mg/ml bovine serum albumin, 0.1 mg/ml catalase, 0.1 mg/ml trypsin, 1 mM Tris-HCl, pH 7.5, and 0.05 M NaCl. The reaction was stopped by the addition of 1 ml of 1 M KH2PO4, pH 5.0.

Immunohistochemistry—Light microscopic immunohistochemical procedures were performed as described previously (29). Briefly, tissues
Fig. 1. Sequence alignment of P3H family members. Human (H), mouse (M), and chicken (C) sequences of prolyl 3-hydroxylase family members were aligned using Vector NTI software. Identical residues across all sequences are shown in white with black shading, whereas black with grey shading indicates identity across some sequences. Protein family members are designated "1", "2", or "3" based on sequence homologies across species. GenBank™ accession numbers for the sequences are as follows: human P3H1 (leprecan), AF097432; mouse P3H1, AAH24047; chicken P3H1, AY463528; human P3H2 (MLAT4), NP_060662; mouse P3H2, NP_775555; chicken P3H2, AY463529; human P3H3 (GRCB), NP_055077; and mouse P3H3, AY463530. Conserved residues of the active site domains of the prolyl 4-hydroxylases and lysyl hydroxylases are indicated with an asterisk, whereas other conserved residues are indicated with a dot. The repeating CXXXC motif in the amino-terminal half of the proteins is indicated with a "-\( ^{+} \)" sign.
exception to this rule thus far is the hypoxia-inducible factor 1. The only known enzyme which binds to denatured collagen or that is exists in a complex of proteins that associate with unfolded or partially folded collagen in the rER during collagen biosynthesis. Molecules that bound specifically were identified and are now known to perform vital roles in the post-translational modifications and processing of the nascent procollagen molecules, such as two members of the peptidyl prolyl cis-trans isomerase family, cyclophilin B (CYPB) and FKBP65, as well as HSP47 and the collagen P4H and PDI. After a high salt wash to remove loosely bound proteins, proteins bound to gelatin-Sepharose were eluted with low pH buffer (Fig. 2A). In addition to the proteins mentioned above, another protein with an apparent molecular mass of 90 kDa on SDS-PAGE was present in the low pH eluted material. We cloned and sequenced the 90-kDa protein and identified it as the chicken homologue of leprecan or Gros1 and the gene we had cloned from chicken embryos. The names P3H1, -2, and -3 correspond to the human genes leprecan on chromosome 1, MLAT4 on chromosome 3, and GRCB on chromosome 12, respectively. They are classified as such based on identity; for example, the translated human sequences for P3H1 and P3H2 are 46% identical, for P3H1 and P3H3 are 41% identical, and for P3H2 and P3H3 are 38% identical. The chicken P3H3 sequence is not yet complete. P3H1 Binds to Denatured Collagen and Exists in a Complex of Proteins—As reported previously, protein extracts from the rER enriched fraction of 15-day-old chick embryos bind to gelatin-Sepharose (24). This method was initially developed as a functional assay to identify proteins or complexes of proteins that associate with unfolded or partially folded collagen in the rER during collagen biosynthesis. Molecules that bound specifically were identified and are now known to perform vital roles in the post-translational modifications and processing of the nascent procollagen molecules, such as two members of the peptidyl prolyl cis-trans isomerase family, cyclophilin B (CYPB) and FKBP65, as well as HSP47 and the collagen P4H and PDI. After a high salt wash to remove loosely bound proteins, proteins bound to gelatin-Sepharose were eluted with low pH buffer (Fig. 2A). In addition to the proteins mentioned above, another protein with an apparent molecular mass of 90 kDa on SDS-PAGE was present in the low pH eluted material. We cloned and sequenced the 90-kDa protein and identified it as the chicken homologue of leprecan or Gros1 and the gene we had cloned from chicken embryos. The names P3H1, -2, and -3 correspond to the human genes leprecan on chromosome 1, MLAT4 on chromosome 3, and GRCB on chromosome 12, respectively. They are classified as such based on identity; for example, the translated human sequences for P3H1 and P3H2 are 46% identical, for P3H1 and P3H3 are 41% identical, and for P3H2 and P3H3 are 38% identical. The chicken P3H3 sequence is not yet complete.

**RESULTS**

### Prolyl 3-Hydroxylases Are a Novel Family of Proteins

In this study we identify prolyl 3-hydroxylase 1 as a novel rER protein present in chick embryo rER extracts partially purified by affinity chromatography on gelatin-Sepharose. As we have previously reported, proteins from rER extracts partially purified can be selected according to their interactions with gelatin (denatured collagen) (24). Eluted proteins were run on SDS-PAGE and transferred to polyvinylidene difluoride membranes, and bands were cut out for amino-terminal sequencing. Partially purified proteins were also subjected to limited trypsin digestion to obtain internal amino acid sequences (data not shown). Degenerate primers were synthesized, and PCR experiments were performed to obtain gene fragments. After an initial PCR fragment was cloned and sequenced, RACE PCR was used to clone the remainder of the gene. Sequence searches against the human and mouse genomes identified three separate genes as potential orthologs of the chick protein we had cloned. Further analysis of all of the nucleotide and translated sequences demonstrated the presence of three closely related genes in all three species (human, mouse, and chicken), one of which clearly matched the published sequence of leprecan or Gros1 and the gene we had cloned from chicken embryos. The names P3H1, -2, and -3 correspond to the human genes leprecan on chromosome 1, MLAT4 on chromosome 3, and GRCB on chromosome 12, respectively. They are classified as such based on identity; for example, the translated human sequences for P3H1 and P3H2 are 46% identical, for P3H1 and P3H3 are 41% identical, and for P3H2 and P3H3 are 38% identical. The chicken P3H3 sequence is not yet complete. **Fig. 1** is an alignment of the translated amino acid sequence of all three genes from human and mouse and two genes from chicken. The carboxyl-terminal portion of all of the molecules is highly conserved and contains critical catalytic residues shared with the lysyl and prolyl 4-hydroxylase enzymes (Fig. 1, indicated with an asterisk) including the two Fe(II)-binding histidines and an aspartic acid residue. Additionally these enzymes share a positively charged residue (lysine in P4H and arginine in LH) at position +10, with respect to the second iron-binding histidine, which is important for binding the C-5 carboxyl group of 2-oxoglutarate (1). The only known exception to this rule thus far is the hypoxia-inducible factor asparaginyl hydroxylase (FIH) in which the residue binding the C-5 carboxyl group of the 2-oxoglutarate is a lysine in position +15 with respect to the first iron-binding histidine (33, 34). Other conserved residues that are shared across the P4H and LH families are indicated with a dot (•) in the figure. More variations are found in the amino-terminal portion of the molecules across families and species; however, all family members contain four repeats of CXXXX, of unknown function, in that region of the molecule (indicated by a + sign in Fig. 1). Finally, all proteins contain the rER retention signal at their carboxyl-terminal end, indicating that they are likely to be resident ER proteins.

### Affinity Purification of P3H1 and Coimmunoprecipitation of CYPB and CRTAP

Prolyl 3-hydroxylase was purified using an antibody bound to agarose beads. Eluted fractions containing P3H1 from gelatin-Sepharose were pooled and run over the antibody column. The elute from the gelatin-Sepharose step (shown in A) is the starting material for the antibody column. After extensive washes in PBS, an elution was performed with a phosphate buffer, pH 6.0. Reducing SDS-polyacrylamide gels were run and stained with Coomassie Blue. B, the two proteins that specifically eluted (lanes 1–4) were sequenced and determined to be CYPB (at 21 kDa) and CRTAP (at 46 kDa apparent molecular mass). P3H1 was then eluted in the pH 2.5 glycine buffer (lanes 5–8). C, gelatin-Sepharose pooled fractions were also loaded onto the antibody column, and the column was washed in PBS and then eluted directly with the pH 2.5 elution buffer (lanes 1–4). Fractions run on reducing SDS-polyacrylamide gels and stained with Coomassie Blue were shown to contain all three proteins, P3H1, CYPB, and CRTAP, suggesting a specific association between these proteins.
proteins were specifically eluted (Fig. 2B, lanes 1–4). They had apparent mobilities on SDS-PAGE of 21 and 46 kDa. Amino-terminal sequencing of these bands as well as tryptic digests of the protein with a blocked amino terminus (CRTAP) identified these proteins as cyclophilin B (CYPB) and the cartilage-associated protein CRTAP. Purified P3H1 was then eluted with a low pH buffer (Fig. 2B, lanes 5–8). In Fig. 2C the P3H1 antibody column was not washed with the midrange buffer but eluted immediately following the PBS washes. All three proteins eluted simultaneously (CYPB, CRTAP, and P3H1), with P3H1 apparently the most abundant (Fig. 2C, lanes 1–4). These results suggest the likelihood of P3H1 not only associating intracellularly with unfolded collagen molecules but also with other proteins in a specific manner.

**P3H1 Has Prolyl 3-Hydroxylase Activity**—Purified P3H1 from 15-day-old chick embryos was tested for its enzymatic activity using a labeled procollagen substrate (18, 19, 25, 26). The P3H1 enzyme used in these assays was that purified without CRTAP and CYPB (as shown in Fig. 2B, lanes 5–8). The only 3-hydroxyproline residues found in collagens thus far are in the sequence -Gly-3(S/H)4Hyp-4Hyp-Gly- (12–14, 35, 36). It has been reported that 3-hydroxyproline formation is dependent on the presence of 4-hydroxyproline (17, 18), suggesting that the main substrate sequence for 3-hydroxyproline synthesis is -Gly-Pro-4Hyp-Gly-. It was therefore necessary to incubate the procollagen substrate in a large excess of prolyl 4-hydroxylase to ensure the complete conversion of all appropriate prolyl residues to 4-hydroxylproline. In the enzymatic assay used here, the release of tritiated water has been correlated with the formation of 3-hydroxyproline (26) and is used as a direct measure of enzyme activity. Fig. 3A demonstrates the effect of increasing enzyme concentrations (in µl of enzyme) on the formation of tritiated water (THO, measured in dpm), where enzyme activity is essentially linear with enzyme concentration up to a point at which enzyme concentration is saturating (~200 µl.) Amino acid analysis of the purified protein determined this saturating enzyme concentration to be ~11.4 nM final concentration. Enzyme concentrations used in subsequent assays were performed with a concentration of enzyme at which the activity is linearly related to the formation of tritiated water (75 µl of enzyme, which is equal to ~4.3 nm final concentration of enzyme in a 2-ml reaction volume.) Fig. 3B shows the formation of tritiated water as a function of time. The reaction appears to be nearly complete by about 30 min. Fig. 3C shows the effect of varying substrate concentrations on the formation of tritiated water in a double reciprocal plot. Variation of the substrate concentration gave a $K_m$ of 179 µl of substrate/2 ml of reaction volume or 89.5 µl of substrate/ml, which is similar to the $K_m$ value previously determined for the partially purified enzyme (26). As an important control we were not able to detect any prolyl 3-hydroxylase activity using the purified P4H enzyme in these assays, which indicates that there was no nonspecific release of tritiated water. Additionally, the purified P3H1 enzyme did not have any prolyl 4-hydroxylase activity when tested using the method based on the hydroxylat-ion-coupled decarboxylation of 2-oxo[1-14C]glut- arate (25), thereby excluding the possibility of it being both a P3H and a P4H.

**P3H1 Localizes to Tissues That Express Fibrillar Collagens**—The same monoclonal antibody used for the purification of the P3H1 enzyme was used in immunohistochemical staining of 16-day-old chick embryo tissues. Fig. 4A shows the staining of an embryonic chick foot with the antibody 1C10 that recognizes the P3H enzyme; clear staining for P3H1 is seen in the dermis (a), the tendon (b), and cartilage (c). Additional staining of chick cartilage with the same antibody is seen in Fig. 4B, where resting cartilage is in the lower left corner and the hypertrophic zone is in the upper right corner.
FIG. 4. Immunofluorescence in 16-day chick embryos. Antibodies to P3H1 (1C10 in A–E, G, H, J, and L) and fibrillin (201 in F, I, and K) were used to localize the proteins in tissues from chick embryos. A, foot stained with the antibody 1C10. P3H1 is present in dermis (a), tendon (b), and cartilage (c). In B, cartilage again appears to contain P3H1 as well as tendon (C, upper left) but not skeletal muscle (C, lower right). Staining of P3H1 in kidney (D and E) is restricted to the calyx (E) and is not found in kidney tubules or glomeruli, whereas fibrillin staining is strong and localizes to the kidney glomeruli and tubules (F). In the liver, P3H1 staining is restricted to the interlobular septum (H) and is largely absent from the liver parenchyma (G). Staining for fibrillin (I) shows basement membranes around liver sinusoids. Finally, cardiac muscle stained with antibodies to P3H1 (J) shows little to no staining, whereas the antibody to fibrillin (K) shows strong staining in basement membrane regions around the muscle fibers. Panel L shows positive staining in the smooth muscle layer of the aorta with the antibody to P3H1.

shows the staining of skeletal muscle, where the distribution of P3H1 is restricted to tendon. Fig. 4, D–F, shows embryonic chick kidney stained with 1C10 (D and E) and an antibody to fibrillin (201) as a positive control (F). These panels show restricted staining for P3H1 to the calyx (Fig. 4E) but no staining for P3H1 in kidney tubules or glomeruli (Fig. 4D). Fig. 4, G–I, shows staining of embryonic chick liver with 1C10 (G and H) and 201 (I) antibodies. Again the presence of P3H1 appears to be very restricted, in this case to the interlobular septum (Fig. 4H), but is largely absent from liver parenchyma (Fig. 4G). Finally Fig. 4, J and K, shows cardiac muscle stained with 1C10 and 201, respectively. P3H1 does not appear to be present in cardiac muscle but is present in the aorta (Fig. 4L) and pulmonary artery (data not shown). These tissue distribution studies demonstrate the presence of P3H1 in areas where fibrillar collagens are synthesized (dermis, tendon, cartilage, large blood vessels, and connective tissue septae). In tissues such as kidney cortex, liver parenchyma, and skeletal and cardiac muscle where basement membrane collagens predominate, P3H1 does not appear to be abundant, if present at all.

DISCUSSION

In this study we report that the chick homologue of leprecan has prolyl 3-hydroxylase activity in an assay using a labeled procollagen substrate (26), and we hereby designate the enzyme P3H1. We show here that P3H1 belongs to a family of proteins based on sequence alignments and high sequence homologies across three species. All three family members share conserved residues of the 2-oxoglutarate- and iron-dependent dioxygenases.

Previously prolyl 3-hydroxylase was partially purified from rat kidney cortex (19) and initially characterized (18, 19, 26), but no genes or amino acid sequences were ever reported for the protein. It was shown that the enzyme activities of P4H and P3H were separate (17) and that both required 2-oxoglutarate, ascorbate, and Fe(II) for their activities (18, 19, 26). In the present study we have partially purified the P3H1 enzyme using gelatin affinity chromatography, a technique that has previously been used to identify other proteins and complexes of proteins that bind to denatured collagen (23, 24). Using this method, specifically interacting proteins were identified by amino-terminal sequencing; these molecules, for example CYPB, FKBP65, HSP47, P4H, and PDI, are now known to be involved in the post-translational modification and processing of procollagen. In this study we report an additional protein, P3H1, that specifically interacts with gelatin (denatured collagen). After being eluted at low pH from the gelatin affinity resin, P3H1 was further purified by affinity chromatography using a monoclonal antibody that specifically recognizes the P3H1. Interestingly, while purifying the enzyme in the second step we found other proteins, namely CYPB and CRTAP, to interact specifically with P3H1 on the antibody column. When the column was eluted with a buffer of pH 6, CYPB and CRTAP were eluted, whereas P3H1 eluted in the pH 2.5 buffer. Our results suggest that P3H1 forms a tight complex with CYPB and CRTAP; however, their presence is not required for full prolyl 3-hydroxylase activity, because the enzyme assays were performed in their absence and no additional enzyme activity was observed when the assays were performed in the presence of CYPB and CRTAP (data not shown). It is likely that this complex of proteins, P3H1, CYPB, and CRTAP, and possibly other larger complexes interact with unfolded procollagen chains in vivo to achieve a fully folded and assembled collagen molecule inside the cell.

In the present study we measured the amount of prolyl 3-hydroxylase activity as a function of the release of tritiated water. As had been shown previously, enzyme activity was linearly proportional to the amount of enzyme added at low to moderate enzyme concentrations (up to approximately 11.4 nM final con-
the folding and assembly of triple helical collagen. More studies necessary for P3H enzyme activity. The results presented here the presence of other interacting proteins does not appear to be remains to be tested. It is interesting to note that unlike P4H, correlates with the presence of fibrillar collagens, we conclude affinity substrate, and because the P3H1 immunolocalization gen. Because we used a denatured fibrillar collagen as the complex of proteins that specifically bind to denatured colla-

fibrillar collagens and may be present at low levels in other collagen molecules as well. A more careful analysis of the (S)Hyp content in collagens and other proteins is necessary to better determine its abundance, especially relative to 4(R)Hyp, and whether it exists in sequences other than -Gly-3-Hyp-4-Hyp-Gly-. Based on the results presented here, we hypothesize that prolyl 3-hydroxylation of collagens is due to the activity of three distinct gene products. Here we show that P3H1 can be purified from the rER of embryonic chick cells and is present in a complex of proteins that specifically bind to denatured collagen. Because we used a denatured fibrillar collagen as the affinity substrate, and because the P3H1 immunolocalization correlates with the presence of fibrillar collagen, we conclude that P3H1 likely serves to modify the fibrillar collagens. Whether it will also modify basement membrane collagen remains to be tested. It is interesting to note that unlike P4H, which requires the presence of PDI for its enzymatic activity, the presence of other interacting proteins does not appear to be necessary for P3H enzyme activity. The results presented here support the idea that P3H plays an important biological role in the folding and assembly of triple helical collagen. More studies on the three P3H family members and their patterns of expression and enzymatic activity will help to clarify their individual roles in collagen biosynthesis.

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