Cloning of the Human Prolyl 4-Hydroxylase α Subunit Isoform α(II) and Characterization of the Type II Enzyme Tetramer

THE α(I) AND α(II) SUBUNITS DO NOT FORM A MIXED α(I)α(II)β2 TETRAMER

(PDI, EC 5.3.4.1)³ and has PDI activity even when present in the prolyl 4-hydroxylase tetramer (4–6). The PDI polypeptide also has several other functions (1–3, 7, 8).

Prolyl 4-hydroxylase had long been assumed to be of one type only, with no isoenzymes (1–3), but recently an isomerase of the α subunit, termed the α(II) subunit, was cloned from mouse tissues (9). Correspondingly, the previously known α subunit is now called the α(I) subunit. The α(II) subunit was found to form an α(II)₂β₂ tetramer with the PDI/β subunit when the two polypeptides were coexpressed in insect cells. The properties of the new type II enzyme were found to be very similar to those of the type I tetramer, with the distinct difference that it was inhibited by poly(l-proline) only at very high concentrations (9).

The α subunit of prolyl 4-hydroxylase cloned from the nematode Caenorhabditis elegans (10) has been found to have features of both types of mouse α subunit, suggesting that C. elegans may have only one type of α subunit (9). This forms active prolyl 4-hydroxylase in insect cell coexpression experiments with either the C. elegans or the human PDI/β polypeptide, but surprisingly, the enzymes containing the C. elegans α subunit are αβ dimers (10, 11).

We report here that the existence of α(II) subunit mRNA is not limited to the mouse, as a corresponding mRNA is expressed in a variety of human tissues. All the data so far available on the existence of the type II prolyl 4-hydroxylase tetramer are based on insect cell coexpression experiments, but we now demonstrate that this enzyme is indeed present in cultured human fibroblasts and represents about 30% of their total prolyl 4-hydroxylase activity. We also studied whether the α(I) and α(II) subunits can form a mixed α(I)α(II)β₂ tetramer, and whether any differences are found between the type I and II enzymes in their Kᵣ values for various peptide substrates, as the two mouse enzymes differ so markedly from each other with respect to inhibition by poly(l-proline). A new affinity purification procedure was developed that is based on the use of a histidine tag in the N terminus of the PDI/β polypeptide, and this makes it possible to obtain large amounts of any form of the recombinant enzyme by very simple steps.

MATERIALS AND METHODS

Isolation of cDNA Clones—Screening of a human lung agt10 cDNA library (CLONTECH) with BT 14.1, a cDNA clone for the mouse α(II) subunit (9), as a probe yielded one positive clone, H9, among 600,000 recombinants. Rescreening of the same library with H9 as a probe gave 8 positive clones out of 600,000 recombinants. Four of them, L121, L142, L21, and L22, were characterized further.

The abbreviations used are: PDI, protein disulfide-isomerase; His-PDI, PDI containing a histidine affinity tag in its N terminus; PAGE, polyacrylamide gel electrophoresis.

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Nucleotide Sequencing, Sequence Analysis, and Northern Blot Analysis—The nucleotide sequences were obtained by the dideoxy nucleotide chain termination method (12) with T7 DNA polymerase (Pharmacia). Vector-specific or sequence-specific 17-mer primers synthesized in an Applied Biosystems DNA Synthesizer (Dept. of Biochemistry, University of Technology, Sydney) and the sequenced with 5'-blabeled oligonucleotides encoding either the whole human α subunit or PA-58 and PA-59 (13) encoding almost all of the human α subunit. The autoradiography time was 3 days.

Construction of Baculovirus Transfer Vectors and Generation of Recombinant Baculoviruses—To construct a baculovirus transfer vector for the human α subunit, a 5' fragment was amplified from the α-DNA of L142. The cDNA-specific primers used were αINS (5'-TCAGGCGGCGGCACAGCCAGACACTTCCCTC-3'), containing an artificial NotI site, and αIE (5'-GGTTGAGAATTCGCGCTGCA-3'), containing a natural EcoRI site. Polymerase chain reaction was performed under the conditions recommended by the supplier of the Taq polymerase (Promega), and the reactions were cycled 27 times as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 3 min. The product was digested with NotI and EcoRI restriction enzymes to give a fragment that extended from nucleotide 166 to 251. The next fragment was prepared by digesting L142 with EcoRI and SacI. The resulting EcoRI-SacI fragment, covering nucleotides 252-873, was ligated together with the above NotI-EcoRI fragment to the NotI-SacI site of the pBlueScript vector (Stratagene) and termed HuNS-BS. Clone H9 was digested with SacI and PstI to give a fragment encompassing nucleotides 874-1426. Clone L21 was digested with PstI, and the resulting fragment, covering nucleotides 1427-2021, was ligated together with the SacI-PstI fragment to the SacI-PstI site of pBlueScript, and the construct was termed I3-SSP-BS. The NotI-SacI fragment from HuNS-BS, and the SacI-PstI fragment from I3-SSP-BS were then ligated to the NotI-PstI site of pBlueScript, and the construct was termed αI1-human-BS. Finally, the αI1-human-BS was digested with NotI and EcoRV, and the resulting fragment was ligated to the NotI-SmaI site of the transfer vector pVL1392 (14).

A histidine affinity tag was generated in the N terminus of the human PDI/β polypeptide. Six histidine codons were created by polymerase chain reaction downstream of the codon coding for the last amino acid of the signal peptide in a full-length cDNA for the human PDI/β polypeptide (4). The cDNA was digested with EcoRI and BamHI and ligated to pVL1392.

Spodoptera frugiperda Sf9 insect cells (Invitrogen) were cultured in TNN-FH medium (Sigma) supplemented with 10% fetal bovine serum (BioClear) at 27 °C, either as monolayers or in suspension in spinner flasks (BioClear) at 27 °C as determined by the confluent cultures were harvested, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a solution of 0.1 M glycine, 0.2 M NaCl, 50 μM dithiothreitol, 0.1% Triton X-100, 0.01% soybean trypsin inhibitor, and 20 mM Tris-HCl buffer, pH adjusted to 7.5 at 4 °C, and centrifuged at 10,000 × g for 30 min. The resulting supernatants were analyzed by nondenaturing 8% PAGE performed under the conditions recommended by the manufacturer of the gel (Bio-Rad). Total prol 4-hydroxylase activity was measured in aliquots of the supernatants, and the type II enzyme activity in aliquots of the supernatants that had been passed through small poly(ethylene) columns.

Assay of Type II Prol 4-Hydroxylase Activity in Tissues of 17-Day-Old Chick Embryos—Calvaria, sternum, tendon, and liver tissues from 17-day-old chick embryos and whole chick embryos were homogenized in a solution of 0.1 M glycine, 0.2 M NaCl, 50 μM dithiothreitol, 0.1% Triton X-100, 0.01% soybean trypsin inhibitor, and 20 mM Tris-HCl buffer, pH adjusted to 7.5 at 4 °C, and centrifuged at 10,000 × g for 30 min. Aliquots of the supernatants were then used to assay the enzyme activities as above.

Protein Purification and N-terminal Amino Acid Sequence Analysis—The type II prol 4-hydroxylase resulting from coinfection with the I3-SSP-BS and PDI/β polypeptides was first purified by gel filtration on a DEAE-cellulose column (DE52 Whatman) and two gel filtrations. Sf9 insect cells harvested 72 h after infection were washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a solution of 0.1 M glycine, 0.1 M NaCl, 10 μM dithiothreitol, 0.1% Triton X-100, and 0.01 M Tris, pH 7.8, supplemented with 22 mM N-ethylmaleimide and 2.2 mM phenylmethylsulfonyl fluoride, and centrifuged. The supernatant was mixed with 1.3 and 0.1 M Tris, pH 7.8, and applied to a DEAE-cellulose column (20 ml) equilibrated with 0.1 M glycine, 0.05 M NaCl, 10 μM dithiothreitol, and 0.01 M Tris, pH 7.8 (buffer A). Unbound material was washed off with the same buffer, and the bound proteins were eluted with an increasing salt concentration generated by mixing 75 ml of buffer A and 75 ml of a solution of 0.1 M glycine, 0.4 M NaCl, 10 μM dithiothreitol, and 0.05 M Tris, pH 7.8 (buffer B). The 5-ml fractions were collected, and the activity was pooled, concentrated by ultrafiltration (Millipore), and applied to a 1.5 × 90-cm Ultrigel AcA34 (Serva) column, equilibrated, and eluted with a solution of 0.1 M glycine, 0.25 M NaCl, 10 μM dithiothreitol, and 0.01 M Tris, pH 7.8. Fractions of 3.5 ml were collected, and their absorbance at 280 nm was measured. Several fractions were analyzed by 8% SDS-PAGE and assayed for prol 4-hydroxylase activity. The 3.5 ml sample pool was used for further purification of the type II enzyme activity, pooling concentrated by ultrafiltration, and 1.5 ml applied to a 95-cm Bio-Gel A-0.5m fine (Bio-Rad) column equilibrated and eluted as above. Fractions of 2.5 ml were collected, analyzed by 8% SDS-PAGE, and assayed for prol 4-hydroxylase activity.

The type I and II prol 4-hydroxylase tetramers and the PDI/β polypeptide containing the histidine affinity tag were also purified by a procedure consisting of a metal chelate affinity chromatography and a gel filtration step. Insect cells expressing type I or type II prol 4-hydroxylase or the His-PDI polypeptide were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a 0.3 M NaCl, 0.1 M glycine, 0.1% Triton X-100, and 0.01 M Tris buffer, pH 7.8. The supernatant was applied to a ProBond column (Invitrogen) equilibrated with 0.3 M NaCl, 0.1 M glycine, and 0.01 M Tris buffer, pH 7.8. Unbound material was washed off with the same buffer, and the bound proteins were eluted with a 100 mM imidazole, 0.3 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, and 0.01 M Tris buffer, pH 7.8. Fractions containing the eluted proteins were pooled and applied to a 2.5 × 95-cm Bio-Gel A-0.5m fine (Bio-Rad) gel filtration column, equilibrated, and eluted with a 0.3 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, and 0.01 M Tris buffer, pH 7.8. Fractions containing the prol 4-hydroxylase tetramer and the PDI/β polypeptide containing the histidine affinity tag were purified by a procedure involving a metal chelate affinity chromatography and a gel filtration. Insect cells expressing type I or type II prol 4-hydroxylase or the PDI polypeptide containing the histidine affinity tag were also purified by a procedure involving a metal chelate affinity chromatography and a gel filtration step. Insect cells expressing type I or type II prol 4-hydroxylase or the His-PDI polypeptide were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a 0.3 M NaCl, 0.1 M glycine, 0.1% Triton X-100, and 0.01 M Tris buffer, pH 7.8. Fractions containing the eluted proteins were pooled and applied to a 2.5 × 95-cm Bio-Gel A-0.5m fine (Bio-Rad) gel filtration column, equilibrated, and eluted with a 0.3 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, and 0.01 M Tris buffer, pH 7.8. Fractions containing the prol 4-hydroxylase tetramer and the PDI/β polypeptide containing the histidine affinity tag were purified by a procedure involving a metal chelate affinity chromatography and a gel filtration. Insect cells expressing type I or type II prol 4-hydroxylase or the His-PDI polypeptide were harvested 72 h after infection, washed with a solution of 0.15 M NaCl and 0.02 M phosphate, pH 7.4, homogenized in a 0.3 M NaCl, 0.1 M glycine, 0.1% Triton X-100, and 0.01 M Tris buffer, pH 7.8. Fractions containing the eluted proteins were pooled and applied to a 2.5 × 95-cm Bio-Gel A-0.5m fine (Bio-Rad) gel filtration column, equilibrated, and eluted with a 0.3 M NaCl, 0.1 M glycine, 10 μM dithiothreitol, and 0.01 M Tris buffer, pH 7.8. Fractions containing the prol 4-hydroxylase tetramer and the PDI/β polypeptide containing the histidine affinity tag were purified by a procedure involving a metal chelate affinity chromatography and a gel filtration step.
For the N-terminal sequencing the type II prolyl 4-hydroxylase was run on 8% SDS-PAGE under reducing conditions and blotted onto a polyvinylidene difluoride membrane. The membrane was stained with 0.1% Coomassie R-250 in 50% methanol, and the band corresponding to the \( \alpha \) (II) subunit was cut off. The N-terminal sequence was determined using an Applied Biosystems model 477A on-line 120 liquid pulse protein sequencer.

Enzyme Activity Assays—Prolyl 4-hydroxylase activity was assayed by a method based either on the hydroxylation-coupled decarboxylation of 2-oxo[1-14C]glutarate or on the formation of hydroxy[14C]proline in protocollagen, a biologically prepared, [14C]proline-labeled protein substrate consisting of nonhydroxylated \( \alpha \) chains of chick type I pro-collagen (17). \( K_m \) values were determined by varying the concentration of one substrate in the presence of fixed concentrations of the second while the concentrations of the other substrates were held constant (18). The pure type II prolyl 4-hydroxylase tetramer was used as an enzyme source for the \( K_m \) determinations.

RESULTS

Isolation of cDNA Clones—To isolate cDNA clones for the human \( \alpha \) (II) subunit, a human lung cDNA library was screened using BT14.1, a cDNA for the mouse \( \alpha \) (II) subunit (9), as a probe. One positive clone, H9, which codes for the central region of the cDNA, was obtained. Rescreening of the cDNA library with H9 as a probe gave 8 positive clones, 4 of which were characterized further.

Amino Acid Sequence of the Human \( \alpha \) (II) Subunit and Its Comparison with the Human \( \alpha \) (I) and Mouse \( \alpha \) (II) Subunits—Sequences—The cDNA clones cover 2194 nucleotides of the corresponding mRNA (these cDNA sequences are not shown, but have been deposited in the GenBank/EMBL Data Bank with accession numbers U90441) and encode a 535-amino acid polypeptide, including a putative signal peptide of 21 residues (Fig. 1). Sequencing of the N terminus of the polypeptide indicated that its first amino acid is glutamate (Fig. 1). The first amino acid of the mouse \( \alpha \) (II) subunit was previously thought to be tryptophan (9), based on the computational parameters of von Hejne (19), but it now seems more likely that this is likewise glutamate, as such a result would also be compatible with the computational parameters.

The human \( \alpha \) (II), human \( \alpha \) (I), and mouse \( \alpha \) (II) polypeptides are very similar in size, \( \alpha \) (I) being three amino acids longer (Fig. 1). All three polypeptides contain two potential attachment sites for asparagine-linked oligosaccharides, but the position of the C-terminal site in the \( \alpha \) (II) and \( \alpha \) (I) subunits

FIG. 1. Comparison of the amino acid sequence of the \( \alpha \) (II) subunit of human prolyl 4-hydroxylase with those of the human \( \alpha \) (I) and mouse \( \alpha \) (II) subunits. Numbering of amino acids begins with the first residue in the processed \( \alpha \) (II) subunit. The amino acids of the human \( \alpha \) (I) and mouse \( \alpha \) (II) subunits are shown by dots when they are identical to those of the human \( \alpha \) (II) subunit and by letters when they are nonidentical. Dashes (–) are introduced for maximal alignment of the polypeptides. The cysteine residues are circled, and the potential attachment sites for asparagine-linked oligosaccharides are boxed.
differs by 4 residues (Fig. 1). The five cysteine residues present in the human, mouse, and chick α(I) and the C. elegans α subunit are all conserved in the human α(II) subunit, but the latter contains an additional cysteine, which is also present in the mouse α(II) subunit (Fig. 1).

The derived amino acid sequence of the human α(II) subunit shows 64% identity and 81% similarity to the sequence of the human α(I) subunit and 93% identity and 96% similarity to the sequence of the mouse α(II) subunit. The identity of the subunits is not distributed equally, being highest within the C-terminal domains (Fig. 1).

Expression of the α(II) and α(I) Subunit mRNAs in Various Human Tissues—Expression of the two types of α subunit mRNA in various human tissues was studied by Northern hybridization. The sizes of the mRNAs for the human α(I) and α(II) subunits are 2.3 and 3.0 kilobases, respectively (Fig. 2). The mRNA for the α(II) subunit was found to be expressed in a variety of tissues, but distinct differences were found relative to the expression pattern of that for the α(I) subunit, in that the relative expression level of the latter was much higher in the skeletal muscle, liver and kidney (Fig. 2).

The α(I) and α(II) Subunits Do Not Form α(I)α(II)β2 Tetramers—To study whether the α(I) and α(II) subunits can form α(I)α(II)β2 mixed tetramers in addition to the α(I)β2 type I and α(II)β2 type II tetramers (1–3, 9), both types of α subunit were expressed in insect cells together with the PDIβ polypeptide. The α(I)β2 tetramer is effectively inhibited by poly(L-proline) (1, 2) and becomes bound to poly(L-proline) affinity columns (20). The α(I)β2 tetramer differs distinctly from the type I enzyme in that it is inhibited by poly(L-proline) only at very high concentrations (9). It thus can be expected that the type II enzyme will not become bound to poly(L-proline), due to the presence of the α(I) subunit, or to remain unbound.

Sf9 insect cells were coinfected with baculoviruses coding for either the α(I) subunit or the α(II) subunit together with viruses coding for the PDIβ polypeptide, and a third set of cells was infected with all three viruses. The cells were harvested 72 h after infection, and Triton X-100-soluble proteins were analyzed by PAGE performed under nondenaturing conditions. When the cells were coinfected with viruses coding for either the α(I) subunit or the α(II) subunit together with a virus coding for the PDIβ polypeptide, a type I or type II enzyme tetramer was formed, the mobilities of these two types of tetramer being essentially identical (Fig. 3A, lanes 1 and 4). An enzyme tetramer was likewise formed when the cells were infected with all three viruses (Fig. 3A, lane 7).

Western blotting with monoclonal antibodies specific to the isolated α(I) and α(II) subunits was used to distinguish between the types of tetramer. The antibody to the α(I) subunit stained the type I tetramer (Fig. 3B, lane 1) but not the type II tetramer (Fig. 3B, lane 4), whereas the antibody to the α(II) subunit stained the type II tetramer (Fig. 3C, lane 4) but not the type I tetramer (Fig. 3C, lane 1).

The type I tetramer became efficiently bound to a poly(L-proline) affinity column, as no enzyme could be detected in the column effluent (Fig. 3, A and B, lanes 2), and could be eluted with poly(L-proline) (Fig. 3, A and B, lanes 3). The type II tetramer was found in the column effluent (Fig. 3, A and C, lanes 5), and no additional amounts could be eluted from the column with poly(L-proline) (Fig. 3, A and C, lanes 6). When the tetramer formed during infection with viruses coding for both types of α subunit was studied as above, a Coomassie-stained enzyme band was seen in both the column effluent (Fig. 3A, lane 8) and the eluate (Fig. 3A, lane 9). The band in the column effluent could be stained by the antibody to the α(II) subunit (Fig. 3C, lane 8) but not to α(I) (Fig. 3B, lane 8), thus ruling out the presence of α(I)α(II)β2 in the effluent. The band in the column eluate could be stained by the antibody to the α(I) subunit (Fig. 3B, lane 9) but not to α(II) (Fig. 3C, lane 9) thus ruling out the presence of α(I)α(II)β2 in the eluate. The absence of α(I)α(II)β2 cannot be due to the low level of infection of Sf9 cells by three viruses simultaneously, as we have recently shown that these cells can be efficiently infected by three viruses (21).

Presence of the Type II Prolyl 4-Hydroxylase Tetramer in Cultured Human WI-38 Fibroblasts and HT-1080 Cells—Confluent cultures of human lung fibroblasts (WI-38) and fibrosarcoma cells (HT-1080) were homogenized, and Triton X-100-soluble proteins were analyzed by PAGE performed under nondenaturing conditions. Western blotting using ECL with monoclonal antibodies specific to the α(II) and α(I) subunits (as demonstrated above) Bands corresponding to both types of enzyme tetramer were found in extracts from both cell types (Fig. 4). Quantification of the Western blots using ECL which had been standardized against known amounts of both types of enzyme tetramer indicated that the amount of type II enzyme is lower than that of type I enzyme in both cell types, being about 30% of total prolyl 4-hydroxylase (details not shown).

Ratio of Type II Enzyme Activity to Total Prolyl 4-Hydroxylase Activity in Human WI-38 Fibroblasts and HT-1080 Cells and Certain Chick Embryo Tissues—Total prolyl 4-hydroxylase activity, i.e. the sum of the type I and type II enzyme activities, was measured in Triton X-100 extracts from cell or tissue...
homogenates using proline-labeled protocollagen as a substrate. The activity of the type II enzyme was measured by passing aliquots of Triton X-100 extracts through poly(L-proline) affinity columns and determining the enzyme activity in column effluents. The values were corrected for dilution, and the type I enzyme activity was estimated by subtracting the type II activity from the total enzyme activity in confluent cultures of human lung fibroblasts and HT-1080 cells (details not shown). The corresponding percentage in Triton extracts from homogenates of 17-day-old whole chick embryos was 10%, and the percentages in Triton extracts from homogenates of chick embryo tissues were 13% in calvaria, 8% in sternum, 10% in tendon, and 5% in liver (details not shown).

**Purification of the Human Type II Prolyl 4-Hydroxylase Tetramer**—As the type II enzyme does not become bound to poly(L-proline), this enzyme could not be purified by the standard affinity column procedure (20, 22) developed for type I. It was therefore initially purified using an ion-exchange chromatography procedure consisting of DEAE-cellulose chromatography and two gel filtrations. The enzyme purified by this procedure was pure as judged by Coomassie staining (A) or Western blotting using an anti-human a(II) subunit monoclonal antibody (B) or anti-mouse a(II) subunit monoclonal antibody (C). The arrows marked 4-PH indicate the enzyme tetramers formed.

tramer with the His-PDI polypeptide (Fig. 6, A and B, lanes 1) as efficiently as with the wild-type PDI/β polypeptide (Table I). The Triton X-100 extracts were then applied to a ProBond column (Invitrogen), the unbound material was washed off with the column equilibrium buffer, and the bound proteins were eluted with imidazole. No enzyme tetramer was found in the flow-through fractions (Fig. 6, A and B, lanes 2), whereas both types of enzyme tetramer and the His-PDI dimer and monomer were present in the column eluates (Fig. 6, A and B, lanes 3). The enzyme tetramers (Fig. 6, A and B, lanes 4) could then be separated from the His-PDI polypeptide by gel filtration, during which the latter was converted from dimers to monomers (Fig. 6, A, lanes 5). The final preparations of both types of enzyme tetramer were also pure when analyzed by SDS-PAGE and Coomassie staining (Fig. 6C).

**Catalytic Properties of the Human Type II Prolyl 4-Hydroxylase Tetramer**—In agreement with data on mouse type II prolyl 4-hydroxylase (9), the $K_m$ values of the human type II enzyme for Fe$^{2+}$, 2-oxoglutarate, and ascorbate were identical to those of the human type I enzyme (Table II). A marked difference was found between the two human enzymes in inhibition by poly(L-proline); however, the $K_i$ values of the type II enzyme for poly(L-proline), $M_i$, 7700, being about 200 times greater than those of the type I enzyme and those for $M_i$, 44,000 about 1000 times greater (Table II). Small but significant differences were found between the human type II and type I enzymes in their $K_m$ values for three peptide substrates, in that all these values were 3–6 times greater in the case of the type II enzyme than for the type I enzyme (Table II). Additional experiments (details not shown) demonstrated that the type II enzyme, like the type I enzyme, did not catalyze any formation
Human Type II Prolyl 4-Hydroxylase

DISCUSSION

The data reported here indicate that the existence of an mRNA for the α(II) subunit of prolyl 4-hydroxylase is not limited to the mouse (9), as an mRNA coding for a highly similar α(II) subunit was also found in human tissues. Furthermore, the present data indicate that the α(II) subunit is translated into the corresponding polypeptide in human cells. The α(II) subunit mRNA was found to be expressed in a variety of tissues, but distinct differences were found in the expression patterns of the α(II) and α(II) subunits between tissues.

Quantification of the proportions of the two types of prolyl 4-hydroxylase tetramer by Western blotting in cultured human WI-38 lung fibroblasts and HT-1080 fibrosarcoma cells indicated that the type II tetramer represents about 50% of the total enzyme protein in these two cell types. Correspondingly, about 50% of the total prolyl 4-hydroxylase activity in extracts from these two cell types was found in the flow-through fractions of the ProBond column, whereas in the cell type II prolyl 4-hydroxylases, as a fraction of the total enzyme activity was found to pass through the poly(t-proline) affinity column, suggesting that the α(II)ββ₂ tetramer represents about 30% of the total prolyl 4-hydroxylase activity. The type II prolyl 4-hydroxylase is also likely to be present in chick embryo tissues, as a fraction of the total enzyme activity was found to be present in the αββ₂ tetramer assembly, but the C-terminal domains of the α subunits, which show the highest degrees of amino acid sequence identity between the α(I) and α(II) subunits and the C. elegans α subunit (9, 10), are known to contain residues involved in the binding of all the cosubstrates to a catalytic site (24, 25). It seems probable that the regions involved in tetramer assembly contain some sequences that prevent incorporation of the α(I) and α(II) subunits into the same molecule.

Although no information is available on the sequences in the α subunits that are critical for tetramer assembly, several observations suggest that in the case of the PDI/β subunit some such sequences are located close to the C-terminal domain of the polypeptide (11, 26). The present data demonstrate that the N terminus of the PDI/β polypeptide is not critical for tetramer assembly, as the His-PDI polypeptide was found to form an active prolyl 4-hydroxylase as readily as the wild-type PDI/β polypeptide. Our additional experiments have demonstrated that the His-PDI polypeptide also efficiently forms an αβ dimer with the C. elegans α subunit (2). The histidine-tagged forms of poly(4-hydroxylase) appear to offer an excellent source of the enzyme for simple large scale purification in experiments such as attempts at crystallization.

Poly(t-proline) has been regarded as a highly effective competitive inhibitor with respect to the polypeptide substrate of prolyl 4-hydroxylases from all vertebrate sources studied, and an efficient polypeptide substrate for all plant prolyl 4-hydroxylases (1–3). It is therefore highly surprising that the human and mouse (9) type II prolyl 4-hydroxylases are inhibited by poly(t-proline) only at very high concentrations. This property of the type II enzyme agrees with that reported for the recombinant C. elegans α subunit (10, 11). As these findings suggest that distinct differences are likely to exist in the structures of the polypeptide binding sites of various prolyl 4-hydroxylases, a comparison was made here between the Km values of the human type I and type II enzymes for three peptide substrates: the polypeptide (Pro-Pro-Gly)₁₀ which is the most commonly

| Cosubstrate, substrate, or inhibitor | Constant | Km or Ki |
|-------------------------------------|----------|---------|
|                                     | α(II)ββ₂ | α(II)ββ₂ |
| Fe²⁺                               | 6        | 4       |
| 2-Oxoglutarate                     | 22       | 22      |
| Ascorbate                           | 340      | 330     |
| (Pro-Pro-Gly)₁₀                    | 18       | 95      |
| GVPQV                               | 22,000   | 7,800   |
| Protocollagen                       | 1.1      | 0.2     |
| Poly(t-proline), M, 7,000           | 95       | 0.5     |
| Poly(t-proline), M, 44,000          | 20       | 0.02    |

TABLE II

Km values of pure human type II and type I prolyl 4-hydroxylases for cosubstrates and various peptide substrates and Km values for poly(t-proline)

a Gly-Val-Pro-Gly-Val.

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used synthetic peptide substrate for prolyl 4-hydroxylase, the pentapeptide Gly-Val-Pro-Gly-Val, which has been introduced as a model peptide for elastin (28), a protein that also contains 4-hydroxyproline (1–3), and protocollagen, a biologically prepared collagenous substrate for the enzyme. Small but significant differences were found between the type I and type II enzymes in these experiments, in that the $K_m$ values for all three peptide substrates with the type II enzyme were about 3–6 times those with the type I enzyme. Nevertheless, these differences are very small when compared with the at least 200–1000-fold differences between their $K_I$ values for poly(L-proline).

Mutations have been characterized in the genes for many types of collagen and for lysyl hydroxylase, a collagen hydroxylase closely related to prolyl 4-hydroxylase in its catalytic properties (3, 29–32). No mutations have been identified in the gene coding for one type of collagen and for lysyl hydroxylase, a protein that also contains the $\alpha(I)$ subunit of prolyl 4-hydroxylase, and due to the central role of this enzyme in the synthesis of all collagens, such mutations have generally been assumed to be lethal. The present data indicating the presence of two isoforms of prolyl 4-hydroxylase $\alpha$ subunit in human tissues raises the possibility, however, that mutations in the gene coding for one type may not be lethal, especially if cells are capable of up-regulating the expression of the other type in cases when one type is inactive.

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