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Comparison Between Avian and Human Prolyl 4-Hydroxylases: Studies on the Holomeric Enzymes and Their Constituent Subunits

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Abstract Prolyl 4-hydroxylase, a key enzyme in collagen biosynthesis, catalyzes the conversion of selected prolyl residues to *trans*-hydroxyproline in nascent or completed pro-α chains of procollagen. The enzyme is a tetramer composed of two nonidentical subunits, designated α and β. To compare the enzyme and its subunits from different sources, the chick embryo and human placental prolyl 4-hydroxylases were purified to homogeneity and their physicochemical and immunological properties were determined. Both enzymes were glycoproteins with estimated apparent molecular weights ranging between 400 and 600 kDa. Amino acid and carbohydrate analyses showed slight differences between the two holomeric enzymes, consistent with their deduced amino acid sequences from their respective cDNAs. Human placental prolyl 4-hydroxylase contained more tightly bound iron than the chick embryo enzyme. Immunodiffusion of the human placental enzyme with antibodies raised against the purified chick embryo prolyl 4-hydroxylase demonstrated partial identity, indicating different antigenic determinants in their tertiary structures. The enzymes could be separated by high-resolution capillary electrophoresis, indicating differential charge densities for the native chick embryo and human placental proteins.

Electrophoretic studies revealed that the human prolyl 4-hydroxylase is a tetrameric enzyme containing two nonidentical subunits of about 64 and 62 kDa, in a ratio of approximately 1 to 2, designated α and β, respectively. In contrast, the chick embryo α and β subunit ratio was 1 to 1. Notably, the human α subunit was partially degraded when subjected to electrophoresis under denaturing conditions. Analogously, when the chick embryo enzyme was subjected to limited proteolysis, selective degradation of the α subunit was observed. Finally, only the α subunit was bound to Concanavalin A demonstrating that the α subunits of prolyl 4-hydroxylase in both species were glycosylated. Using biochemical techniques, these results demonstrated that the 4-*trans*-hydroxy-*L*-proline residues in human placental collagens are synthesized by an enzyme whose primary structure and immunological properties differ from those of the previously well-characterized chick embryo enzyme, consistent with their recently deduced primary structures from cDNA sequences.

Key words: collagen biosynthesis, capillary electrophoresis, glycoprotein, protein disulfide isomerase, p55-thyroid hormone binding protein, phosphoinositide-specific phospholipase C, glycosylation site binding protein, iodothyronine-5′-monodeiodinase

Prolyl 4-hydroxylase (prolyl-glycyl-peptide, 2-oxoglutarate dioxygenase, 4-hydroxylating; EC 1.14.11.2) catalyzes the cotranslational and post-translational conversion of selected prolyl residues to 4-*trans*-hydroxy-*L*-proline. This reaction is carried out in nascent or completed pro-α chains of fibrillar procollagen, basement membrane procollagen, and in collagen-like domains of a variety of peptides and proteins [for reviews, see Cardinale and Udenfriend, 1974; Prockop et al., 1976; Kivirikko and Myllylä, 1980; Kivirikko et al., 1989; Guzman et al., 1990a]. Most of

Abbreviations used: Con A, Concanavalin A; cpm, counts per minute; DEAE, diethylaminoethyl; dpm, disintegrations per minute; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; kDa, kilodalton; OPA, o-phthalaldehyde; PDI, protein disulfide isomerase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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4-*trans*-hydroxy-*L*-proline in vertebrate tissues is found in collagen and its presence significantly contributes to the thermal stability of the triple-helical conformation of collagen [Berg and Prockop, 1973a; Jimenez et al., 1973; Rosenbloom et al., 1973].

Prolyl 4-hydroxylase, a mixed function oxygenase, requires, in addition to the peptide substrate, α-ketoglutarate, ascorbate, ferrous ions and molecular oxygen as cosubstrates for optimal catalysis [Hayaishi et al., 1975; Siegel, 1979; Kivirikko et al., 1989; Guzman et al., 1990a]. Serum albumin, dithiothreitol, catalase, nucleoside triphosphate [Takeda et al., 1977], specific chelating agents [Takeda et al., 1979a], bleomycin [Takeda et al., 1979b], and thymol [Peterkofsky et al., 1980] also stimulate prolyl 4-hydroxylase activity. The precise mechanisms of action for these substances remain unclear.

Prolyl 4-hydroxylase was purified initially by conventional chromatographic procedures from various sources including chick embryo homogenates [Halme et al., 1970; Pänkäläinen et al., 1970], neonatal rat skin extracts [Rhoads and Udenfriend, 1970], and neonatal mouse skin homogenates [McGee et al., 1971]. Subsequently, the high affinity of prolyl 4-hydroxylase for its polypeptide substrates [Juva and Prockop, 1969] was utilized to develop an affinity chromatographic procedure for enzyme purification [Berg and Prockop, 1973b]. The affinity column was prepared with collagen from *Ascaris* cuticle, a collagen with an unusually low content of hydroxyproline [McBride and Harrington, 1967]. The elution of prolyl 4-hydroxylase from the column was accomplished by using a synthetic peptide $(Pro\text{-}Gly\text{-}Pro)_n$, a substrate analogue for the enzyme. To date, several modifications of the affinity purification procedure have been described [see Guzman et al., 1990a]. Using these procedures, prolyl 4-hydroxylase has been purified to homogeneity from a variety of sources including chick embryo [Berg and Prockop, 1973b; Tuderman et al., 1975; Kedersha and Berg, 1981; Nietfeld and Kemp, 1980, 1981; Nietfeld et al., 1981; Ishimaru et al., 1982], fetal human tissues [Kuutti et al., 1975], rat carrageenin granulomas [Takeda et al., 1977], neonatal rabbit skin [Chichester et al., 1976, 1979; Chichester and Fuller, 1980], cultured mouse fibroblasts [Kao and Berg, 1979; Kao and Lee Chou, 1980], rat fetal [Takeda et al., 1979a] and neonatal tissues [Risteli et al., 1976; Chen-Kiang, et al., 1977; Rokowski et al., 1981], human placenta [Guzman et al., 1982; Nagai and Oka, 1985; Koivu et al., 1987], and the foot of the marine mussel *Mytilus edulis L.* [Marumo and Waite, 1987].

Purified prolyl 4-hydroxylase from chick embryo was found to be a tetramer of 240 kDa composed of two nonidentical subunits, termed α and β, of 64 and 60 kDa, respectively [Cardinale and Udenfriend, 1974; Prockop et al., 1976; Berg et al., 1979; Kivirikko et al., 1980; Guzman et al., 1990a]. Comparative studies of prolyl 4-hydroxylase from chick embryo [Berg and Prockop, 1973b; Tuderman et al., 1975], fetal human tissues [Kuutti et al., 1975], and neonatal rabbit skin [Chichester et al., 1979] demonstrated similar, if not identical, electrophoretic profiles, cofactor requirements, and amino acid compositions. Thus, it has been generally assumed that the prolyl 4-hydroxylases from these species were essentially identical to the chick embryo tetrameric enzyme. Comparative studies of the deduced amino acid sequences from the recently isolated cDNAs, encoding for the α and β subunits of prolyl 4-hydroxylase, from chick embryos and human placenta revealed that the α and β subunits were unique, although significant homology was retained for each subunit between species [Kao et al., 1988; Nakazawa et al., 1988; Parkkonen et al., 1988; Helaakoski et al., 1989; Bassuk et al., 1989]. However, detailed comparisons of the purified protein structures (i.e., physicochemical properties) have not been performed as yet.

Interest in the subunit structure of human prolyl 4-hydroxylase has been intensified recently following reports that the prolyl 4-hydroxylase β subunit has at least seven distinct biological functions [for recent reviews, see Freedman, 1989; Kivirikko et al., 1989, 1990; Guzman et al., 1990a]. This conclusion was based on the observation that the predicted amino acid sequences of the human prolyl 4-hydroxylase β subunit, the monomeric murine protein disulfide isomerase (PDI) (Pihlajaniemi et al., 1987), and the monomeric human thyroid binding protein p55 (Yamauchi et al., 1987; Cheng et al., 1987; Obata et al., 1988; Parkkonen et al., 1988] were essentially identical. In addition, molecular cloning and deduced-cDNA amino acid sequence studies have demonstrated partial similarities to phosphoinositide-specific phospholipase C (Bennett et al., 1988), a glycosylation site binding protein component of oligosaccharyl transferase [Geetha-Habib et al., 1988],

iodothyronine-5′-monodeiodinase [Boado et al., 1988], and the estrogen-binding domain of the estrogen receptor [Tsibris et al., 1988]. These findings indicated that the β subunit may function differently as a monomer than when associated with the α subunit of prolyl 4-hydroxylase. Therefore, it has become increasingly important to further characterize the subunit structure of human prolyl 4-hydroxylase and the properties of its subunits. Moreover, such studies may provide insight into the metabolic regulation and function of the α and β subunits of the enzyme. The findings that the α subunit is selectively degraded in vitro could be an important in vivo mechanism for regulating the amount of functional holomeric prolyl 4-hydroxylase.

In this communication, we demonstrate the subunit structure of human prolyl 4-hydroxylase as well as various physicochemical properties of its α and β subunits. In addition, we demonstrate that prolyl 4-hydroxylase purified from normal human placentae differs slightly from the well-characterized chick embryo enzyme in primary structure as well as in other physicochemical and immunological properties.

MATERIALS AND METHODS

Materials

Fresh, full-term placentae were obtained from normal mothers within 30 min after delivery, the umbilical cords were removed, and the tissues were stored at −30°C. Chick embryos from white chickens were purchased from Shamrock Poultry (North Brunswick, NJ). New Zealand white rabbits were from Perfection Breeders (Douglasville, PA). *Ascaris* lumbricoides worms (variation *suis*), having as common habitat pig intestines, were obtained from a local slaughterhouse. Poly(L-proline) type II (molecular weight of 1,000 to 9,000), PMSF, sodium thyocyanate, EDTA (disodium salt), soybean trypsin inhibitor, iodoacetate, N-ethylmaleimide, p-aminobenzamidine-HCL, methyl cellulose, methyl-α-*D*-mannopyranoside, *D*-(−)arabinose, *D*-(+) glucosamine, *D*-(+)galactosamine, 2,2′-dipyridyl, 2-mercaptoethanol, 3-mercaptopropionic acid, urea, silver nitrate, α-ketoglutarate, ascorbate, catalase, and Coomassie brilliant blue R were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-cellulose (DE-52) was from Whatman, (Clifton, NJ). [4-^{3}H]-Proline (30 Ci/nmol), [1-^{14}C]ketoglutaric acid (sodium salt), N-[acetyl-^{3}H]acetylated-Concanavalin A (25 Ci/nmol), protosol, and Aquasol-2 were obtained from New England Nuclear (Boston, MA). Bromphenol blue, acrylamide, and N,N′-methylenebisacrylamide were purchased from Eastman Kodak Co. (Rochester, NY). Nonidet P-40 was from Bethesda Research Lab. (Rockville, MD). Molecular weight standards and Sephacryl S-300 (superfine) were obtained from Pharmacia Inc., (Piscataway, NJ). Amberlite MB3 was purchased from Chemical Dynamics Corp. (South Plainfield, NJ). Agarose was from Marine Colloids Div., FMC Corp. (Rockland, ME). Complete Freund's adjuvant was obtained from Grand Island Biological Co. (Grand Island, NY). Poly (*L*-prolyl-*L*-glycyl-*L*-prolyl) (molecular weight 5,200) was purchased from Miles Laboratories, Inc. (Elkhart, IN). Diaflo ultrafiltration membranes were from Amicon Corp. (Lexington, MA). Dowex 50 W-X8 (200–400 mesh, hydrogen form), Dowex AG 1-X2 (200–400 mesh, acetate form), Agarose Bio-Gel A-1.5 m (200–400 mesh), Agarose Bio-Gel A-150 m (100–200 mesh), and dithiothreitol were obtained from Bio-Rad Laboratories (Richmond, CA). Standards of alditol acetate derivatives of neutral sugars (Mix-1 and Mix-2), microreaction vessels (5 ml), and a coiled glass column (O.D. ¼ inch; I.D. 4 mm; length, 6 feet) packed with 3% SP-2340 and 100/120 Supelcoport were purchased from Supelco (Bellefonte, PA). Fused-silica capillary columns were obtained from Scientific Glass Engineering (Austin, TX) and from Polymicro Technologies (Phoenix, AZ). Other materials were in the highest purity available from commercial sources.

High performance liquid chromatography was carried out with two different systems: 1) modular components were obtained from Waters Associates (Milford, MA), and 2) an integral component (HP-1090) was from Hewlett-Packard Co. (Avondale, PA). Capillary zone electrophoresis was performed with an automated apparatus similar to that previously described [Guzman et al., 1990b]. The system is a full-featured instrument (Princeton Biochemicals, Inc., Princeton, NJ) that includes an autosampler or autoloader; a modified on-column UV, variable-wavelength, detection system; a direct current regulated high-voltage power supply (30 kV, 1,000 μA) used in the positive voltage mode, modified to obtain a reverse field polarity capability; a fraction collector; an on-line degassing system; and an automated cleaning device to clean the capillary column. In this instrument, the capillary is housed in a cassette-cartridge constructed to allow a flow of recirculating liquid for temperature control of the capillary column. The liquid is in

direct contact with the capillary. Data collection for quantitation of electropherographic peaks was carried out with an integrator model D-2500 Chromato-Integrator (Hitachi Instruments, Inc., Danbury, CT). Electropherograms were generated with a strip chart recorder model L-6512 (Linseis Inc., Princeton Junction, NJ) at 20 cm/h and 1 mV output. The microcolumn liquid chromatography detector cell was modified with a slit to fit the capillary column. The polymer coating of the vitreous silica tubing was partly burned out at the detection point of the tube to make an on-column UV cell. The modification was made to obtain a higher amplifier gain and a shorter response time. Bare fused silica capillaries (I.D. 75 μm; O.D. 375 μm) were used.

METHODS

Purification of Prolyl 4-Hydroxylase From Chick Embryos and Human Placentae

For the purification of prolyl 4-hydroxylase, 13-day-old chick embryos and full-term human placentae were used. The frozen placentae were thawed at 4°C for one day. In a few experiments, fresh placentae were cut into small pieces, frozen in liquid nitrogen, and taken directly for enzyme purification.

The enzymes were purified by the method described by Guzman et al. [1982] with slight modifications. About 800 g wet weight of chick embryos were homogenized in two separate batches, or 4 kg wet weight of thawed human placentae were homogenized in 8 separate batches in 1.5 volumes of 0.01 M Tris-HCl buffer (pH 7.3) at 25°C, containing 0.1 M NaCl, 0.3% (v/v) Nonidet P-40, 0.02% (w/v) sodium azide, 10 μM DTT, and 0.1 mM PMSF (homogenization buffer). All procedures were performed at 4°C. Homogenization was carried out in a Waring blender for 90 sec at high speed and the homogenate was centrifuged at 20,000*g* for 40 min. The supernatant, containing about 40 mg/ml of protein, was filtered through glass wool and cheesecloth, and the pellet was discarded.

The 20,000*g* supernatant containing about 40 g of chick embryo protein or 200 g of human placental protein was applied to a DEAE-cellulose column (10.3 × 20 cm) at a flow rate of 500 ml per h. The column was equilibrated with 0.01 M Tris-HCl buffer (pH 7.3) containing 0.1 M NaCl, 0.02% (w/v) sodium azide, 10 μM DTT, and 0.1 M PMSF (enzyme buffer). The column was washed with enzyme buffer and then eluted in a single step with enzyme buffer containing 0.6 M NaCl. The enzyme-containing effluent was then applied to the affinity column (3.0 × 45 cm) which contained reduced and carboxymethylated *Ascaris* cuticle collagen [Berg and Prockop, 1973b, 1976], at a flow rate of 20 ml per h. After application of the enzyme preparation, the column was washed exhaustively with enzyme buffer and then eluted with 90 ml of enzyme buffer containing 3.0 mg per ml of poly(L-proline) [Tuderman et al., 1975]. In a few experiments, the substrate analogue $(Pro\text{-}Gly\text{-}Pro)_n$ (1.0 mg per ml) was used to elute prolyl 4-hydroxylase from the affinity column. Further separation of poly(L-proline) or $(Pro\text{-}Gly\text{-}Pro)_n$ and proteins was accomplished by anion-exchange chromatography [Kedersha and Berg, 1981; Guzman et al., 1982]. About 150 ml of the affinity column effluent were collected and then applied to a DEAE-cellulose column (1.5 × 25 cm) which was equilibrated with enzyme buffer. The column was washed exhaustively with enzyme buffer and then eluted with 80 ml of enzyme buffer containing 0.6 M NaCl. Aliquots of 2 ml were collected at a flow rate of 40 ml per h and the effluent of the column was monitored for optical density at 280 nm and prolyl 4-hydroxylase activity. Finally, the enzyme preparation was subjected to HPLC as described below.

HPLC Column Operation and Calibration

Either modular or integral HPLC systems were used (see Materials). Two Protein I-250 columns (Waters Associates; 7.8 × 30 cm) packed with 10 μm silica based media to which a hydrophobic moiety was bound were used; the columns were connected in tandem. Routinely, a guard precolumn dry packed with I-125 gel filtration media (50 μm) was employed. Aqueous phases were filtered (0.22 μm Millipore filters) and degassed before use. The mobile phase was 10 mM Tris-HCl (pH 7.3) containing either no salt or 500 mM NaCl. Chromatography routinely was performed at a flow rate of 1.0 ml per min at a pressure of 1,200 psi at 25°C. Sample injection volume was between 50 and 150 μl of the filtered purified enzyme preparations or purified protein standards used for molecular weight calibration.

Prolyl 4-Hydroxylase Assay

Prolyl 4-hydroxylase activity was assayed by the tritium release method of Hutton et al. [1966] as modified by Cutroneo et al. [1974]. The [^{3}H]labeled protocollagen substrate was prepared from chick embryo calvaria as described

by Miller [1975]; however, [4-^{3}H]proline was used instead of radioactive lysine. Tritium release was linearly related to the time of incubation for up to 40 min. With incubation times of 30 min, the release of tritium was proportional to the amount of 20,000*g* supernatant over the range of 0.5 to 6 mg of protein or purified enzyme over the range of 1 to 8 μg of protein. The cpm of tritium released was 3 to 30 times the radioactivity of the blank (about 30–60 cpm) which was prepared by incubating the substrate without enzyme. With different substrate preparations, the maximal amounts of [^{3}H]H_2O varied from 2% to 8% of the total tritium in the substrate. The resulting tritiated water formed was collected by vacuum distillation and 0.8 ml of a total volume of 1.0 ml was dissolved in Aquasol-2 and radioactivity was measured by liquid scintillation spectroscopy at an efficiency of 25%. Protein concentrations were determined by the method of Lowry et al. [1951].

Hydroxylation of [^{3}H]Protocollagen by Prolyl 4-Hydroxylase

[^{3}H]Labeled protocollagen was prepared as described above and the hydroxylation of [^{3}H]-protocollagen was determined by assessing the formation of radioactive total hydroxyproline [Cutroneo et al., 1972; Farjanel et al., 1980], or more specifically and sensitively, by assessing the formation of the fluorescent 3- and 4-hydroxyproline isomer derivatives when protocollagen was hydrolyzed, oxidized, and tagged to *o*-phthalaldehyde [Liebes et al., 1984].

In the first method, approximately 60,000 cpm of [^{3}H]protocollagen were incubated aerobically at 30°C for 30 min with 8 μg of affinity purified chick embryo or human placental prolyl 4-hydroxylase in the presence of 0.3 μmol of ferrous ammonium sulfate, 4.9 μmol of ascorbate, 0.6 μmol of α-ketoglutarate, 0.25 mg of bovine liver catalase, 10 mg of bovine serum albumin, and 220 μmol of Tris-HCl (pH 7.5) in a total volume of 1.0 ml. After the incubation, the enzymic reaction was terminated by the addition of 1.0 ml of 12 N HCl. The mixture was adjusted to 5% (v/v) phenol [Waite and Benedict, 1984] and the tubes were sealed under vacuum. The hydrolysis was then carried out for 16 h at 100°C in a block heater (Supelco Inc., Bellefonte, PA). After hydrolysis, the samples were centifuged at 10,000*g* for 15 min and the supernatants were neutralized with 4 N NaOH. The samples were chromatographed on a Dowex 50 W-X8 column (1 × 20 cm), washed with 40 ml of distilled water followed by elution with 1 N HCl, during which time 2.0 ml fractions were collected. Hydroxyproline was present in the first 40 ml of flow through and proline was eluted in the second 40 ml. The recovery of hydroxyproline from the column ranged from 80% to 85% as assessed in experiments with radioactive 4-hydroxyproline. Material eluting in the first 40 ml was evaporated to dryness and applied to Whatman No. 1 chromatography paper for descending chromatography with butanol:acetic acid:water (4:1:5 v/v/v) as the solvent system. The radioactivity applied to the paper migrated to the same R_F as purified unlabeled 4-hydroxyproline.

The fluorescent method was carried out using the conditions described above for the radioactive method with the following modifications: after sample hydrolysis, the hydrolysates were evaporated in a freeze-dry centrifuge (Speed Vac, Savant Instruments Inc., Hickville, NY). The dried hydrolysates then were hydrated with 0.015 M sodium phosphate buffer (pH 7.0) at room temperature and centrifuged at 10,000*g* for 5 min to remove any particulate material. The supernate was removed and diluted 1:500 with the same buffer. Then the sample was further diluted 1:40 with sodium hypochlorite (1% w/v sodium hypochlorite in 0.2 M borate buffer, pH 10.0, at room temperature). The samples were then capped and heated at 50°C for 8 min to allow complete oxidation of the amino acids, which were then reacted with an equal volume of *o*-phthalaldehyde reagent and the derivatization process was carried out for 4 min at 25°C. The OPA reagent was prepared at least 2 days in advance and consisted of 0.5% (w/v) OPA, 0.5% (v/v) 2-mercaptoethanol, 0.1% (v/v) Brij in 1.0 M borate buffer (pH 10.0 at 25°C). The derivatized samples were immediately analyzed by HPLC using a reverse phase C18 column (4.5 × 250 mm) obtained from SOTA chromatography (Crompond, NY). The samples were resolved during a 25 min cycle using a linear elution gradient of 0% to 75% (v/v) acetonitrile in 0.01 M phosphate buffer (pH 7.0). The hydroxyproline isomer standards were converted to OPA derivatives as described above, including 4-*trans*-hydroxy-*L*-proline (Calbiochem, San Diego, CA) and 3-*trans*-hydroxy-*L*-proline prepared by the procedure of Szymanovicz et al. [1978].

Preparation of Antibodies and Immunodiffusion

Antibodies directed against pure chick embryo prolyl 4-hydroxylase were raised in rabbits

as described previously [Berg et al., 1972]. The antibodies were purified by immunoadsorption on a column containing pure prolyl 4-hydroxylase covalently bound to 1% agarose (Bio-Gel A-150, BIO-RAD). The column was then eluted with 3 M sodium thiocyanate in 0.01 M sodium phosphate buffer (pH 7.0) and stored at −30°C in 0.5 ml aliquots before use.

Immunodiffusion was carried out in plastic Petri dishes using a modification of the Ouchterlony method [Ouchterlony, 1958] with 1% (w/v) agarose in 0.1 M Tris-HCl buffer (pH 8.3) containing 0.1 M boric acid and 0.003 M disodium EDTA salt.

Polyacrylamide Slab and Cylindrical Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in SDS was carried out by the method of King and Laemmli [1971] as described elsewhere [Guzman et al., 1978]. All samples were reduced by adding 2-mercaptoethanol to a final concentration of 5% (v/v) and then boiled for 3 min. Alternatively, addition of the less odorous compound, 3-mercaptopropionic acid, to a final concentration of 7% (v/v) was as effective as 2-mercaptoethanol. The gels were either 12% or 15% and were stained with Coomassie brilliant blue or by a modified silver-staining procedure [Wray et al., 1981]. Some of the slab gels electrophoresed under denaturing conditions were stained, destained, cut into strips, and then scanned at 500 nm in a Gilford Model 240 spectrophotometer.

Cylindrical gel electrophoresis of native and denatured human and chick embryo prolyl 4-hydroxylases also was performed. For gel electrophoresis under native conditions, the purified enzymes were concentrated with an Amicon PM-30 membrane and electrophoresed in 7% polyacrylamide cylindrical gels at pH 8.3 as described previously [Guzman et al., 1976]. The gels were stained with Coomassie brilliant blue R, destained, and then scanned at 500 nm. In a few experiments, both human and avian enzymes were electrophoresed under native or denaturing conditions in cylindrical gels and then were incubated at 4°C for 24 h with 5 μCi of [acetyl-^{3}H]Con A as described elsewhere [Guzman et al., 1976]. After incubation, the gels were washed with either buffer or buffer-containing methyl-α-*D*-manno pyranoside, and sliced into 1 mm slices by freezing the gels in pulverized dry ice. The samples were then swelled with Protosol solution and cooled, and the radioactivity was determined in a liquid scintillation spectrophotometer.

High Performance Capillary Electrophoresis

Buffer-filled open-tubular capillaries represent an attractive alternative to gels as a medium in which to conduct zone electrophoresis. Untreated fused-silica (quartz) capillaries (75 μm I.D. × 100 cm), with a total volume of 4.4 μl, were used. The columns were further surface-modified or deactivated before use by slowly pumping a solution of 0.2% (w/v) methylcellulose at a flow rate of 10 μl per min [Hjertén and Zhu, 1985]. The capillary columns were then equilibrated with 0.05 M sodium tetraborate buffer (pH 8.3). Methylcellulose was used to reduce the electroosmotic flow of buffer generated when very high voltages were applied across the narrow bore capillaries and to minimize adsorption of the solutes onto the capillary walls.

Affinity purified chick embryo or placental prolyl 4-hydroxylase (3–5 nl of a solution containing about 3–4 mg per ml) was "injected" electrokinetically (7,000 V for 12 sec) into the capillary column by inserting the capillary column and a platinum-iridium electrode into a 1.5 ml microcentrifuge plastic tube reservoir containing approximately 100 μl of enzyme(s) solution. Following injection of the purified enzyme(s), the voltage was switched off. Both the capillary column and the platinum-iridium electrode were connected to a motor-driven, electronic-controlled arm. This automated process of inserting such small amounts of samples into the capillary column allows very accurate reproducibility of the system, particularly for quantitative purposes. The other reservoir contained 50 ml of 0.05 M sodium tetraborate buffer (pH 8.3) with a second platinum-iridium electrode. The straight length of the fused capillary column connected the anodic buffer reservoir with the electrically grounded cathodic buffer reservoir.

The capillary column and the platinum electrode were then transferred automatically to a 1.5 ml microcentrifuge plastic reservoir containing 1.0 ml of 0.05 M sodium tetraborate buffer (pH 8.3). The electrophoretic voltage for separation of the proteins consisted of a potential gradient of about 220 volts per cm (22,000 volts), about 60 microamps, to a maximum running time of about 25 min. All operations were carried out at constant temperature by thermostating the capillary at 25°C using a liquid cooling

system (Endocal Model rte-110D, Neslab Instruments, Inc., Portsmouth, NH). In this instrument, the capillary was housed in a cassette-cartridge device configured in a coiled shape to allow a flow of the recirculating liquid (Guzman et al., 1990b). It is important that the water used in the separation procedure was deionized and triply distilled, and that the buffer solutions were passed through a 0.22 μm pore size filter unit (Schleicher and Schuell, Keene, NH) and extensively degassed before use. In order to obtain high reproducible values, the capillary column was washed in between injections and regenerated as described by Lauer and McManigill [1985].

Peptide Mapping of the Chick Embryo and Human Placental Enzymes

Linear peptide maps of each of the enzymes were obtained using previously described methods [Gross, 1967; Kasper, 1975; Seyer and Kang, 1977]. About 2 mg of lyophilized prolyl 4-hydroxylase from chick embryo or human placenta were suspended in 0.5 ml of 70% formic acid and 10 mg of cyanogen bromide. The solution was then flashed with nitrogen and the reaction was allowed to proceed for 8 h at 25°C. The incubation mixture was stopped by freezing and lyophilization was performed three times. In order to eliminate formic acid completely, the sample was resuspended in 2 ml distilled water after each lyophilization. The lyophilized samples were then suspended in sample buffer [Guzman et al., 1976] and electrophoresis was performed in 15% SDS polyacrylamide slab gels as described above. After staining and destaining of the gels, gel strips were cut and scanned at 500 nm as described above.

Chromatography of Prolyl 4-Hydroxylase on Concanavalin A-Sepharose

Approximately 0.9 mg of affinity purified chick embryo or human placental prolyl 4-hydroxylase was dissolved in 0.2 M NaCl, 0.2 M glycine, and 0.01 M Tris-HCl buffer (pH 7.8). The samples were slowly applied to individual columns (0.6 × 2.0 cm) of Con A-Sepharose. Each column was washed with 20 ml of the above buffer and eluted with the same buffer containing 0.2 M methyl-α-*D*-mannopyranoside. The eluate was monitored by optical density at 280 nm and by measuring enzymic activity in each fraction.

Amino Acid and Carbohydrate Composition Analyses

For amino acid analysis, samples were hydrolyzed with 6 N HCl. The mixtures were adjusted to 5% phenol [Waite and Benedict, 1984] and the tubes were sealed under vacuum. The hydrolysis was then carried out for 16 h at 100°C and the analyses were performed with a Beckman 119 amino acid analyzer.

For carbohydrate analysis, samples were hydrolyzed with 1 N HCl and then processed to obtain the alditol acetate derivatives of the neutral sugars as described previously [Berg et al., 1979]. Gas-liquid chromatography was performed using a Hewlett-Packard Model 5830 gas-liquid chromatograph equipped with a flame ionization detector. The oven temperature was maintained at 225°C throughout the run and the injector and detector temperatures were 250°C. *D*-(−)-Arabinose was used as an internal standard in all neutral sugar determinations. The elution positions of alditol acetate derivatives of sugars were determined by subjecting a standard mixture of alditol acetate derivatives of neutral sugars to gas-liquid chromatography under identical conditions. Hexosamines were determined after hydrolysis of the samples in 4 N HCl for 12 h at 100°C. Each hydrolyzate was evaporated and then applied to the amino acid analyzer using the conditions described previously [Berg et al., 1979]. Under these conditions both glucosamine and galactosamine were separated.

RESULTS

Electrophoretic Migration of Prolyl 4-Hydroxylases Under Native Conditions

In order to determine the charge density of the two enzymes, affinity purified prolyl 4-hydroxylases from chick embryos and human placentae were subjected to ion-exchange high-performance liquid chromatography, cylindrical gel electrophoresis, and high-resolution capillary electrophoresis. Open-tubular capillary free-zone electrophoresis has been shown to be a technique capable of unusually high separation efficiency [Jorgenson et al., 1988; Karger et al., 1989; Guzman et al., 1989, 1990b]. Conventional polyacrylamide cylindrical gel electrophoresis and ion-exchange high-performance liquid chromatography did not resolve the proteins (data not shown). Only capillary electrophoresis was capable of clearly differentiating between the two holomeric enzymes (Fig. 1), indicating a

different charge density between the two hydroxylases under native conditions. The avian enzyme appears to be more positively charged, migrating toward the negative pole faster than the human placental enzyme.

HPLC Profiles and Determination of the Molecular Weights of Chick Embryo and Placental Prolyl 4-Hydroxylase

Conventional gel filtration chromatography has been used as last step for the purification of placental prolyl 4-hydroxylase [Guzman et al., 1982]. However, the method was slow and the yield of active enzyme was low. Therefore, to facilitate enzyme purification, high performance (size exclusion) liquid chromatography was evaluated (Fig. 2). The improved procedure made it possible to efficiently and consistently obtain homogeneous prolyl 4-hydroxylase from human placentae and chick embryos with more than 95% recovery of enzymic activity and protein. The results were identical whether poly(*L*-proline) or $(Pro\text{-}Gly\text{-}Pro)_n$ was used to elute the enzymes from the affinity column (not shown). However, the apparent molecular weight of both

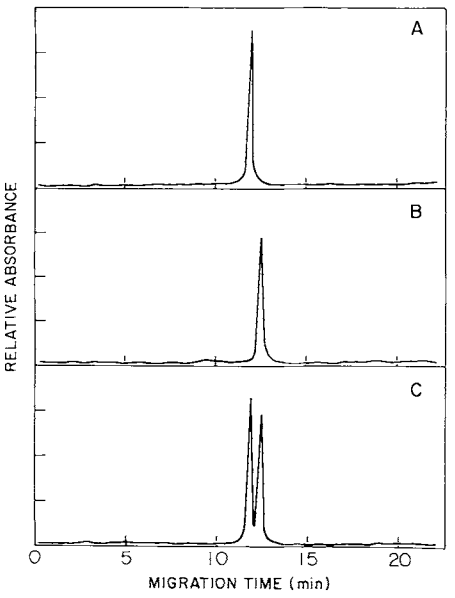

Fig. 1. High-performance capillary electrophoresis of purified prolyl 4-hydroxylase from human placenta and chick embryo homogenates. Buffer-filled open-tubular capillary electrophoresis was carried out as described in the text. Approximately 5 nl of an affinity purified enzyme solution (containing about 4 mg of protein per ml) was electrokinetically injected onto the capillary column. **A:** Chick embryo prolyl 4-hydroxylase. **B:** Human placenta prolyl 4-hydroxylase. **C:** Separation of a mixture of chick embryo and human placental prolyl 4-hydroxylases.

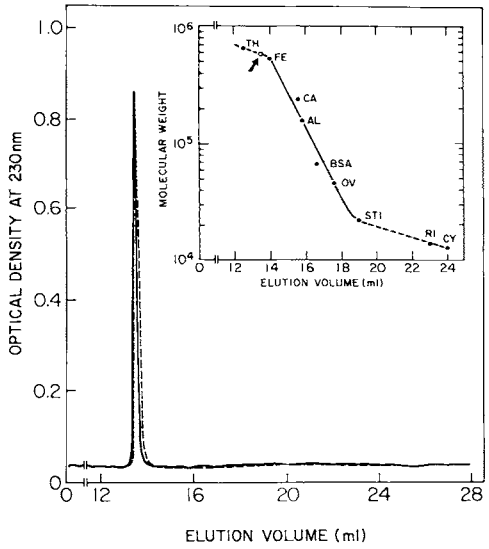

Fig. 2. Gel filtration HPLC of prolyl 4-hydroxylase. The purified enzymes were injected on a I-250 Protein column as described in the text. The mobile phase used here was 10 mM Tris-HCl (pH 7.3) containing no salt. The major protein peak containing all the enzymatic activity was re-injected into the HPLC system. **Solid line:** Human placental prolyl 4-hydroxylase. **Dotted line:** Chick embryo prolyl 4-hydroxylase. **Insert:** Calibration curve for molecular weight standards. TH, thyroglobulin (669,000); FE, ferritin (440,000); CA, catalase (232,000); AL, aldolase (158,000); BSA, bovine serum albumin (67,000); OV, ovalbumin (43,000); STI, soybean trypsin inhibitor (20,100); RI, ribonuclease (13,700); CY, cytochrome C (12,300). The arrow indicates the position of both avian and mammalian prolyl 4-hydroxylases when buffer containing no salt was used.

the placental and chick embryo enzymes was greater than the molecular weight (240 kDa) previously determined for chick embryo prolyl 4-hydroxylase by ultracentrifugation [Cardinale and Udenfriend, 1974; Prockop et al., 1976]. Molecular weights greater than 240 kDa were obtained by conventional size exclusion chromatography for prolyl 4-hydroxylase isolated from various animal [Guzman et al., 1990a] and higher-plant [Bolwell et al., 1985] sources. The only lower molecular weight (40 kDa) holomeric prolyl 4-hydroxylase is from the unicellular and multicellular green algae *Chlamydomonas reinhardii* [Kaska et al., 1987] and *Volvox carteri* [Kaska et al., 1988]. Therefore, it is not apparent whether the high molecular weights obtained here for the chick embryo and placental enzymes (approximately 400 kDa when buffer containing high salt was used, or 600 kDa when salt was omitted from the buffer) reflect aggregation of the proteins or anomalous behavior in size exclusion columns. It is notable that the anomalous motility of mussel prolyl 4-hydroxylase recently was observed when the purified

protein was subjected to size exclusion in the presence of Tris buffers [Marumo and Waite, 1987].

Subunit Composition of Placental Prolyl 4-Hydroxylase

Prolyl 4-hydroxylase from all vertebrate sources studied to date is a tetramer consisting of two different types of enzymatically inactive monomeric subunits, termed α and β. The tetramer from the newborn rat skin and chick embryos have the same subunit composition of $\alpha_2\beta_2$ ($\alpha = \beta = 1$), with a molecular weight of 64 kDa for the α subunit and 60 kDa for the β subunit [Chen-Kiang et al., 1977; Berg et al., 1979]. As shown in Figure 3, human placental prolyl 4-hydroxylase also is composed of two nonidentical subunits, α and β. However, the ratio in which the monomers are present in the placental enzyme is different from that found in the chick embryo or rat enzyme, due to the preferential degradation of the human enzyme α subunit. As shown in Figure 4A, and in agreement with previous observations [Berg and Prockop, 1973b; Berg et al., 1979], the chick embryo prolyl 4-hydroxylase α and β subunits occur in a ratio of about 1 to 1. Figure 4B shows an approximately 1 to 2 ratio for the α and β subunits of the human placental enzyme (the staining intensity of the α subunit derived from the human enzyme is decreased when compared with the staining intensity of the α subunit derived from the avian enzyme). The same ratios were obtained for the placental and chick embryo enzyme subunits when the gels were stained with silver (not shown). Thus, these data indicated that the stoichiometry of the two human subunits differed from those of all other species studied. Although the migration of the α subunits for both enzymes apparently appeared identical, the β subunit of the placental prolyl 4-hydroxylase was observed to migrate slower than that of the β subunit of the chick embryo enzyme.

A consistent observation was that more β subunit was obtained than α subunit when placenta was the enzyme source. This finding suggested the possibility that the α subunit is degraded partially during purification. In order to

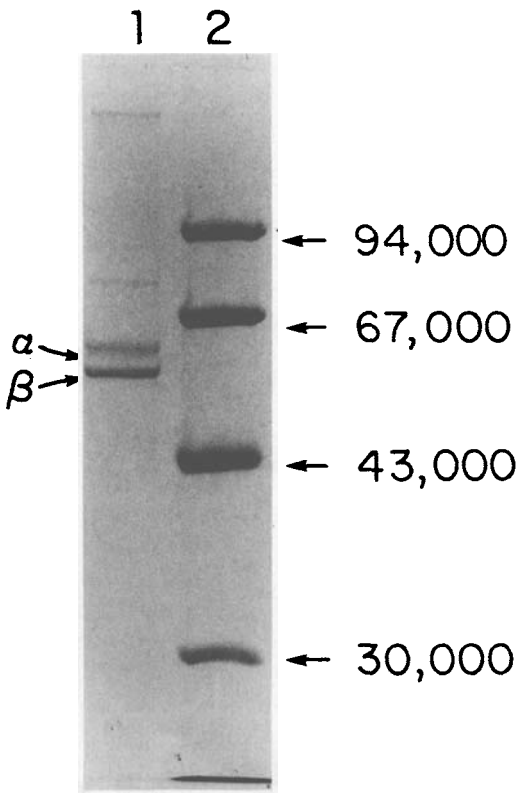

Fig. 3. Sodium dodecyl sulfate polyacrylamide slab gel electrophoresis of purified prolyl 4-hydroxylase from human placenta. See text for details. **Lane 1:** Human placental prolyl 4-hydroxylase. **Lane 2:** Molecular weight standards.

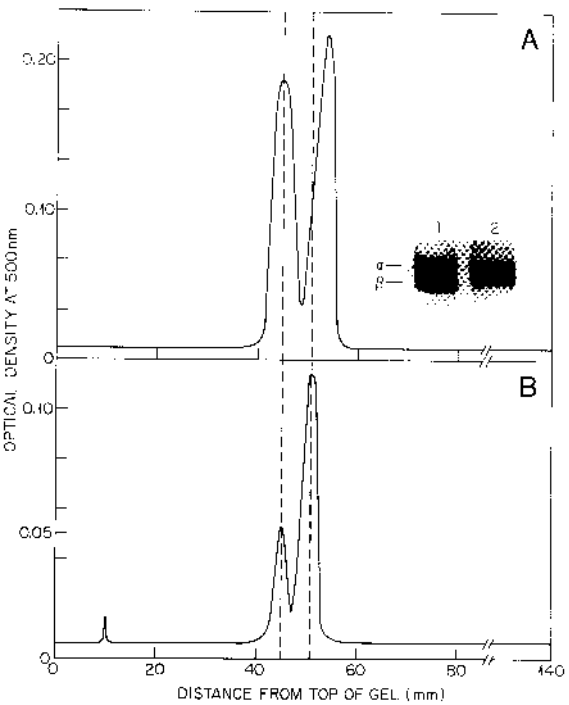

Fig. 4. Stoichiometry and migration properties of the prolyl 4-hydroxylase subunits. Prolyl 4-hydroxylase from human placenta and chick embryo homogenates were purified to homogeneity as described in the text. SDS polyacrylamide slab gel electrophoresis of the purified enzymes was performed. The gel was stained, destained, cut into strips, and then scanned at 500 nm. **A:** Chick embryo prolyl 4-hydroxylase. **B:** Human placental prolyl 4-hydroxylase. **Insert:** Lane 1, chick embryo enzyme; lane 2, placental enzyme.

test this possibility, several experiments were carried out. Fresh full-term placentae obtained within 15 min after delivery were cut at 4°C in small pieces and placed immediately in liquid nitrogen. The thawed samples were homogenized immediately in buffer containing protease inhibitors that are known not to affect enzyme activity, including PMSF (final concentration of 0.1–0.5 mM), soybean trypsin inhibitor (0.01–0.05%) [Kivirikko and Myllylä, 1982], and the protease inhibitors described by Olsen et al. [1976]. Complete purification of the enzyme was carried out within one week. All the above additions were made to avoid proteolysis, particularly of the placental prolyl 4-hydroxylase α subunit. Still, the α to β ratio of the placental enzyme differed from that observed for the chick embryo prolyl 4-hydroxylase (although a significant improvement in preventing degradation of the α subunit derived from the human enzyme was observed). It is possible that some other protease inhibitor(s), not investigated here, may be more effective in preventing α subunit degradation. As a result, the ratios of α subunit to β subunit vary depending upon the degree of degration of the α subunit from the holomeric human enzyme.

A more direct approach to demonstrate the selective degradation of the α subunit was made by exposure of the holomeric enzyme to proteases in vitro. Affinity purified chick embryo enzyme was subjected to proteolytic digestion using *S. aureus* endopeptidase V8 protease. As shown in Figure 5, the α subunit of the chick embryo enzyme was selectively degraded. At low concentrations of *S. aureus* protease, the β subunit was not altered whereas the α subunit was degraded to about 55% of the initial protein. These results suggest that the β subunits of both the avian and human tetrameric enzymes are more resistant to proteolysis than their respective α subunits. At higher concentrations of *S. aureus* protease, the β subunit also was partially degraded (Fig. 5).

Concanavalin A Binding to Prolyl 4-Hydroxylases

Human placental prolyl 4-hydroxylase was analyzed to determine whether it bound to Con A as did the chick embryo [Guzman et al., 1976; Berg et al., 1979] and newborn rat skin [Chen-Kiang et al., 1977] enzymes. Prolyl 4-hydroxylase purified to homogeneity from chick embryo and human placenta sources were subjected to electrophoresis under native conditions and allowed to bind to radioactive Con A (Fig. 6A,B). As shown in Figure 6B, the human enzyme, like the chick enzyme (Fig. 6A), was recovered as a single band of protein when examined by poly-

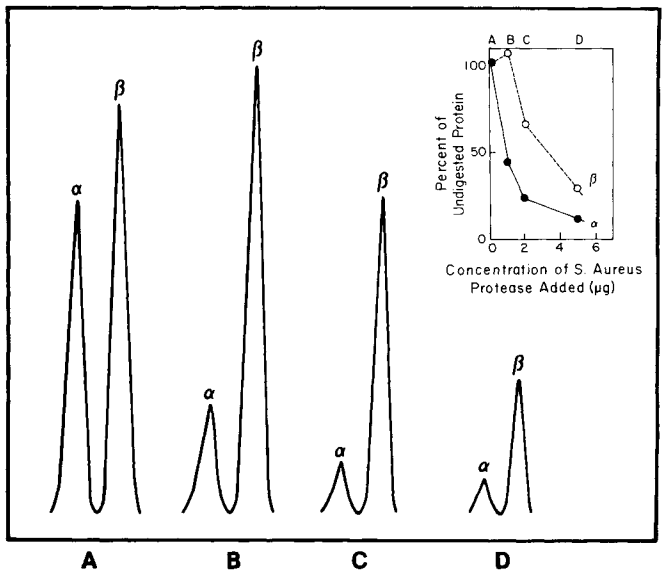

Fig. 5. Selective degradation of the α subunit of the tetrameric prolyl 4-hydroxylase. Prolyl 4-hydroxylase was affinity purified from the chick embryo homogenates as described in the text. The holomeric enzyme was subjected to proteolytic digestion with *S. aureus* V8 protease, and the peptide products were analyzed by electrophoresis using denaturating conditions as indicated in the text. **A:** Undigested prolyl 4-hydroxylase. **B,C,D:** Tetrameric enzyme incubated with 1, 2, and 5 μg of protease, respectively. **Insert:** Comparative proteolytic digestion of the α and β subunits of chick embryo prolyl 4-hydroxylase.

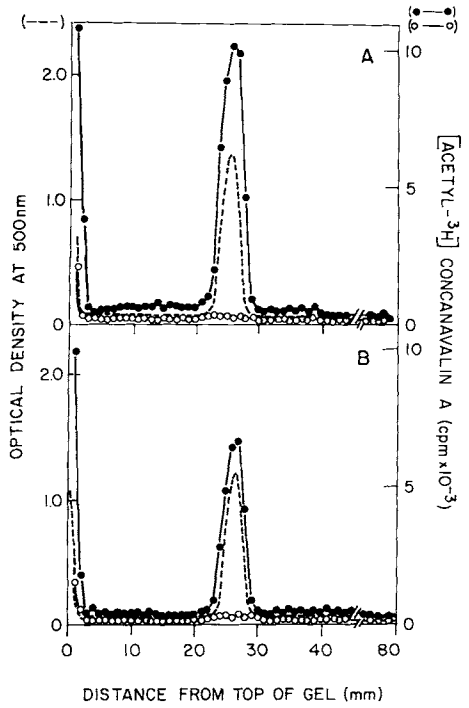

Fig. 6. Native polyacrylamide gel electrophoresis of purified prolyl 4-hydroxylase from avian and human sources. Conditions for electrophoresis were as indicated in text. One gel was stained for protein (···). A second gel was incubated with [acetyl-^{3}H]Con A and then washed with enzyme buffer (●—●). A third gel was incubated with [acetyl-^{3}H]Con A and then washed with enzyme buffer containing 0.2 M metyl-α-*D*-mannopyranoside (○—○). **A:** Chick embryo prolyl 4-hydroxylase. **B:** Human placental prolyl 4-hydroxylase.

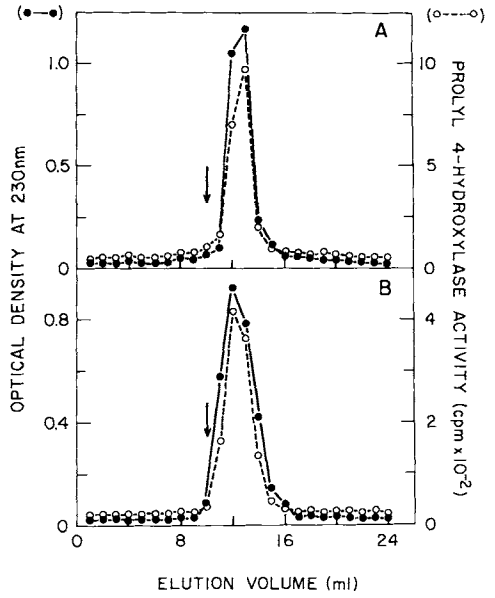

Fig. 7. Con A-Sepharose chromatography of purified prolyl 4-hydroxylase. Prolyl 4-hydroxylases from avian and mammalian sources were chromatographed individually on lectin affinity columns as described in the text. The enzymes then were eluted with buffer containing 0.2 M methyl-α-*D*-mannopyranoside. The effluent fractions were monitored for prolyl 4-hydroxylase activity (○---○) and total protein at 230 nm (●—●). The arrow indicates the beginning of elution. A: Chick embryo prolyl 4-hydroxylase. **B:** Human placental prolyl 4-hydroxylase.

acrylamide gel electrophoresis in Tris-borate buffer (pH 8.3). Previous studies [Guzman et al., 1982] indicated that the protein in the band was enzymatically active and consisted of the holomeric form of the enzyme. When duplicate gels were incubated with [acetyl-^{3}H]Con A, the lectin was bound to the region which contained the enzyme. The binding appeared to be specific, since the radiolabeled Con A was released from the gel containing the human enzyme (Fig. 6B) by washing with a solution containing methyl-α-*D*-mannopyranoside, which specifically binds to the lectin [Poretz and Goldstein, 1970; Lis and Sharon, 1973].

As expected from the binding of [acetyl-^{3}H]-Con A by both the avian and human hydroxylases (Fig. 6A,B), almost 100% of the prolyl 4-hydroxylases were bound to Con A-Sepharose (Fig. 7A,B). When eluted with 0.2 M methyl-α-*D*-mannopyranoside, the recoveries of the chick embryo and placental enzymes from Con A-Sepharose were both about 95% of applied enzymic activity and 100% of applied enzyme protein. When the eluted placental prolyl 4-hydroxylase was electrophoresed on SDS polyacrylamide slab gels, it was found to be composed of two different subunits in a ratio of approximately 1(α) to 2(β) (not shown). These results demonstrated that the human placental prolyl 4-hydroxylase was a glycoprotein, as previously shown for the chick embryo enzyme [Guzman et al., 1976]. In addition, when the denatured enzymes were analyzed for the binding of radiolabeled Con A, only the α subunits of the avian and human enzyme bound to the lectin (Fig. 8A,B).

Amino Acid and Carbohydrate Analyses of Prolyl Hydroxylases

In order to obtain a more accurate amino acid and carbohydrate analysis, the purification scheme of the human placental prolyl 4-hydroxylase was carried out under optimal conditions. The quick-freeze of the placental tissue in liquid nitrogen and the addition of protease inhibitors to the buffers, during the purification,

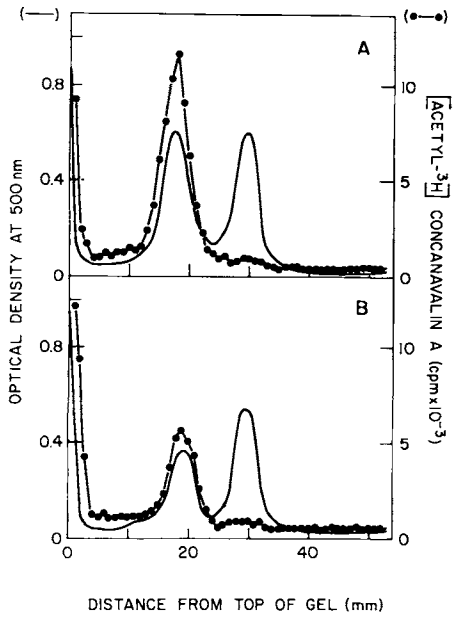

Fig. 8. Denatured polyacrylamide gel electrophoresis of the subunits of purified prolyl 4-hydroxylase from avian and human sources. Conditions for electrophoresis as indicated in the text. One gel was stained for protein (—). A second gel was incubated with [acetyl-^{3}H]Con A and then washed with enzyme buffer (●—●). **A:** Chick embryo prolyl 4-hydroxylase. **B:** Human placental prolyl 4-hydroxylase.

were necessary to prevent partial proteolysis of the holomeric enzyme.

The amino acid composition of the affinity purified human placental prolyl 4-hydroxylase was quite similar to those from other sources (see Table I). However, the human placental enzyme had slightly less glycine, alanine, and arginine. In addition, electrophoresis of cyanogen bromide peptides showed differences in the distribution of methionine residues in the human and avian proteins (not shown).

The carbohydrate content also was determined for the avian and mammalian prolyl 4-hydroxylases. As indicated in Table II, the content of N-acetylglucosamine was virtually the same for both enzymes. However, human placental prolyl 4-hydroxylase contained slightly less mannose than the chick embryo enzyme. These findings might reflect differences in the amount of mannose present in the oligosaccharide moities of the purified placental prolyl 4-hydroxylase α subunit. In addition, there were trace amounts of glucose, galactose, and N-acetylgalactosamine. Most or all of the carbohydrate in the avian enzyme has been located in the α subunit [Chen-Kiang et al., 1977; Berg et al., 1979]. More recently, it has been postulated that the α subunit, isolated from chick embryo tendon cells, exists in two forms which differ in carbohydrate content [Kedersha et al., 1985a,b].

Specificity of Antibody for Prolyl 4-Hydroxylase

Polyclonal antibodies were produced against purified chick embryo prolyl 4-hydroxylase in rabbits. The specific antibodies were purified from the rabbit antisera by immunoadsorption chromatography using chick embryo prolyl 4-hydroxylase conjugate to agarose. As seen in Figure 9, the antibodies reacted with tetrameric form chick embryo prolyl 4-hydroxylase when tested by immunodiffusion, in agreement with previous observations [Berg et al., 1972, 1980]. The polyclonal antibodies directed against the chick embryo enzyme also reacted with the purified placental prolyl hydroxylase. However, the immunological cross-reactivity was of partial identity (Fig. 9), indicating the presence of common and different antigenic determinants. These results indicate the evolutionary divergence of the avian and human prolyl 4-hydroxylases.

Cofactor Requirements for Prolyl 4-Hydroxylases

The maximum activity of purified prolyl 4-hydroxylase, isolated from whole chick embryo homogenates, recovered in the absence of ferrous ions was 75% of control experiments [Ishimaru et al., 1982]. Routinely, lack of exogenous iron in the prolyl 4-hydroxylase assay generated activity values ranging between 25% and 40% of the control activity, depending upon the purity of the enzyme. However, as seen in Table III, purified human placental prolyl 4-hydroxylase did not require exogenous ferrous ions to obtain full enzymic activity, even though the other cofactors were essential for optimal activity. In addition, placental prolyl 4-hydroxylase was less sensitive to inhibition by 2,2'-dipyridyl than chick embryo prolyl 4-hydroxylase. As shown in Figure 10, the human placental enzyme required larger concentrations of the chelating agent (for inhibition of enzyme activity) than the chick embryo enzyme when assayed at the same protein concentrations. These results suggest that the iron in the human holomeric placental prolyl 4-hydroxylase appears to be more tightly bound than in the chick embryo enzyme.

Hydroxylation of Protocollagen by Prolyl 4-Hydroxylase

The two natural isomers of hydroxyproline are 3-hydroxyproline and 4-hydroxyproline. Both

TABLE I. Amino Acid Composition of the Holomeric Prolyl 4-Hydroxylase From Various Sources*

Amino acid	Chick[a]	Chick[b]	Rat[c]	Rabbit[d]	Human[e]	Human[f]	Human[g]
4-Hyp	—	—	—	—	—	—	—
Asx	99	117	118	109	122	117	116
Thr	46	55	53	50	51	54	50
Ser	38	44	45	64	45	53	48
Glx	137	138	143	144	142	138	138
Pro	50	52	43	54	50	42	43
Gly	82	70	72	84	72	68	66
Ala	90	90	89	87	89	79	78
1/2 Cys	—	—	—	—	—	—	—
Val	58	55	59	54	58	58	57
Met	7	—	17	10	—	11	10
Ile	44	43	38	37	45	41	40
Leu	108	104	94	92	98	96	93
Tyr	11	23	30	31	22	34	29
Phe	57	56	53	50	52	54	51
Hyl	—	—	—	—	—	—	—
Lys	93	88	93	73	93	94	90
His	17	17	23	22	19	24	21
Arg	54	48	41	40	42	37	36

*Values are expressed as residues per 1,000 amino acids.
[a]From Berg and Prockop [1973b].
[b]From Tuderman et al. [1975].
[c]From Chen-Kiang et al. [1977].
[d]From Chichester et al. [1979].
[e]From Kuutti et al. [1975].
[f]From Nagai and Oka (dimer) [1985].
[g]From this laboratory.

TABLE II. Carbohydrate Composition of Prolyl 4-Hydroxylase*

Sugar	Chick embryo enzyme	Human placental enzyme
Mannose	35.4	29.8
N-acetyl-glucosamine	3.9	3.8

*The affinity purified avian and mammalian enzymes were hydrolyzed in 0.1 N HCl and the neutral carbohydrate content was determined by gas chromatography as indicated in the text. For hexosamines, the enzymes were hydrolyzed in 0.4 N HCl and determined with an amino acid analyzer. For details see text. Values are expressed as residues per 240 kDa. Results are the mean of duplicate determinations.

hydroxyl groups are *trans* in reference to the carboxyl group located in the C2 position. These imino acids have been used as markers for most fibrillar collagens (4-hydroxyproline) and basement membrane collagens (3-hydroxyproline). In a few avian and mammalian tissues, it has been demonstrated that two separate enzymes are responsible for the synthesis of 4-hydroxyproline and 3-hydroxyproline [see Prockop et al., 1976; Kivirikko and Myllylä, 1982].

In order to demonstrate the specificity of human placental prolyl 4-hydroxylase, it was necessary to analyze the enzymatic products formed when the purified placental enzyme was incubated with purified radiolabeled underhydroxylated procollagen and the appropriate cofactors. As indicated in Figure 11 (insert), a significant amount of radiolabeled hydroxyproline was formed when analyzed by conventional ion-exchange chromatography. However, the separation technique did not resolve the 4-hydroxyprolines that were formed during the enzyme assay in vitro. As shown in Figure 11, 3- and 4-hydroxyproline were separated very clearly when the assay products were subjected to high-performance liquid chromatography using a very selective and sensitive fluorescent procedure for the detection of the imino acids [Liebes et al., 1984]. The results show that enzymic product formed was specifically 4-hydroxyproline when human placental prolyl 4-hydroxylase catalyzed the hy-

Fig. 9. Ouchterlony double immunodiffusion of avian and mammalian prolyl 4-hydroxylases. **Center well:** Purified antibody produced against chick embryo prolyl 4-hydroxylase. **Wells 1,4:** Purified chick embryo enzyme. **Wells 2,3,5,6:** Purified human placental enzyme.

TABLE III. Cofactor Requirements for Prolyl 4-Hydroxylase Activity*

	Prolyl 4-hydroxylase activity (% of total)	
Cofactor	Human placenta	Chick embryo
All cofactors	100	100
Minus α-ketoglutarate	0	0
Minus ascorbate	1	0
Minus molecular oxygen	2	3
Minus ferrous ions	99	45
Minus protocollagen	0	0

*About 8 μg of affinity purified prolyl 4-hydroxylase from chick embryo or human placenta homogenates were tested for essential cofactors as described in the text. Cofactors were omitted one by one from the enzymatic assay. Nitrogen gas was added for the molecular oxygen experiment.

droxylation of substrate protocollagen. The small amount of 3-hydroxyproline present in the assay was probably due to the endogenous amount of 3-hydroxyproline in protocollagen, as demonstrated by control experiments with purified protocollagen (not shown). Virtually the same results were obtained when chick embryo was used as a source of prolyl 4-hydroxylase.

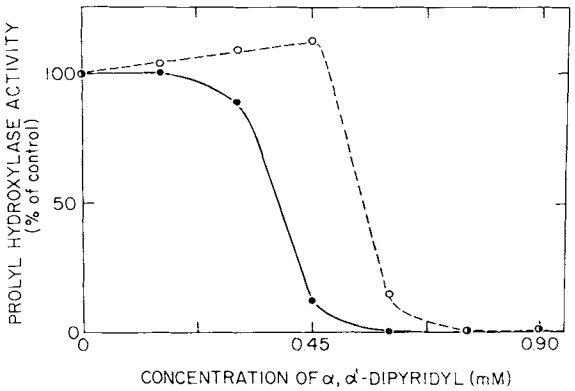

Fig. 10. Inhibition of prolyl 4-hydroxylase activity by 2,2'-dipyridyl. Approximately 8 μg of purified chick embryo or human placental enzyme was assayed for activity in the presence and absence of 2,2'-dipyridyl as described in the text. Human placental prolyl 4-hydroxylase (O---O). Chick embryo prolyl 4-hydroxylase (●—●).

DISCUSSION

Over a dozen genetically distinct collagens have been identified in connective tissues of vertebrates, each having distinct polypeptide subunits [for reviews, see Miller 1985; Mayne and Burgenson, 1987; Vuorio and de Crombrugghe, 1990; Van der Rest and Garrone, 1991]. Hydroxylation of prolyl residues in the Y-position of the repeating X-Y-Gly triplets is essential for newly synthesized proα chains of procollagen to become triple-helical [for review, see Prockop et al., 1979]. Previous work suggested, but never verified chemically, that the physical and kinetic properties of the prolyl 4-hydroxylases were essentially the same across species [Cardinale and Udenfriend, 1974; Prockop et al., 1976; Kivirikko and Myllylä, 1980]. Although significant differences in cofactor requirements have been reported by various laboratories, it has been difficult to compare these results because different tissues, enzymes at various stages of purification, and a variety of methods were used. The first clear indication of structural differences in the prolyl 4-hydroxylase enzymes, from different species, came from the immunological studies of Roberts et al. [1973]. Using polyclonal anti-rat skin prolyl 4-hydroxylase antibodies, they reported partial immunological cross-reactivity with the enzymes derived from mouse, guinea pig, human, and rabbit, and no cross-reactivity with enzymes derived from chick, turtle, frog, earthworm, calf, and sheep. Conversely, Höyhtyä et al. [1984] have demonstrated that when specific monoclonal antibodies were

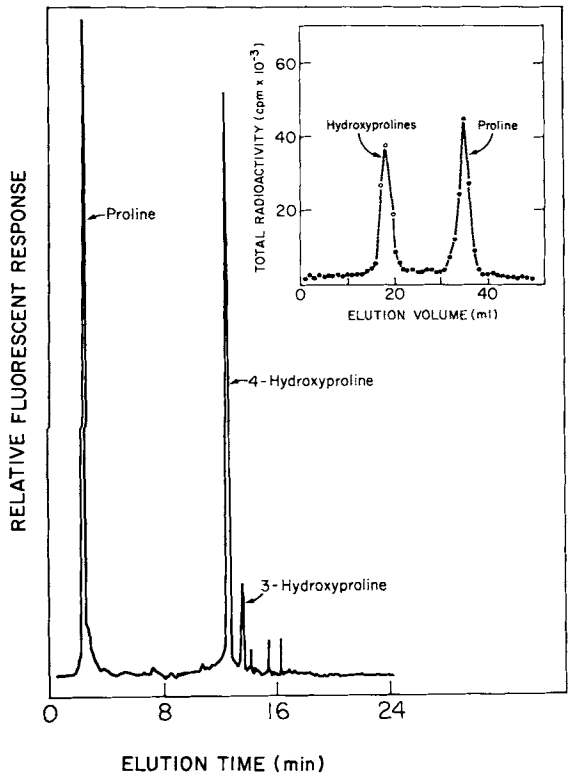

Fig. 11. Identification of the enzymic products after incubation of placental prolyl 4-hydroxylase with radiolabeled protocollagen and appropriate cofactors. The conditions of the reaction mixture are as indicated in the text. Samples were resolved by HPLC using fluorescence methods or by conventional ion-exchange chromatography using radioactivity (**insert**).

generated against human placental prolyl 4-hydroxylase, and when species specificity of the monoclonal antibodies was studied, all the antibodies reacted with the human fibroblasts, whereas none reacted with mouse or chicken cells. Another indication of the structural differences of the enzymes is the fact that the holomeric prolyl 4-hydroxylase purified from the green algae *Chlamydomonas reinhardii* [Kaska et al., 1987] and from *Volvox carteri* [Kaska et al., 1988], was a monomer of 40 kDa whose structure was similar to the α subunit of the tetrameric enzyme, but whose activity also was neutralized by antibodies against the human β subunit [Kaska et al., 1988]. In light of the paucity of data comparing the physicochemical properties of prolyl 4-hydroxylases from various species, especially from mammalian sources, to that of the readily purified and well-characterized chick embryo enzyme, efforts were undertaken to determine and compare the properties of the human placental enzyme.

The purified human placental prolyl 4-hydroxylase was homogeneous as judged by electrophoresis under native and denaturing conditions, by high-performance liquid chromatography, and by a novel procedure for the high resolution of polypeptides, capillary electrophoresis [Guzman et al., 1990b]. The human enzyme was found to differ from the well-characterized chick embryo prolyl 4-hydroxylase in the following properties: the human enzyme bound iron more tightly as reflected by the fact that it was unnecessary to add exogenous iron for optimal activity in the assay system. Also, it was less sensitive to inhibition by the chelator 2,2′-dipyridyl. The native placental enzyme migrated differently from the native chick embryo enzyme when examined by high-performance capillary electrophoresis under native conditions. In addition, the β subunit migrated more slowly than the β subunit of the chick embryo enzyme during electrophoresis under denaturing conditions. Furthermore, the stoichiometry of the subunits differed, the amino acid and carbohydrate composition differed slightly, and the distribution of some amino acids differed as indicated by unique cyanogen bromide peptide maps for the two hydroxylases. Also, human placental and chick embryo prolyl 4-hydroxylases were shown to have both common and different antigenic determinants. Therefore, these data clearly document differences in the primary structure of the subunits of the human and chick embryo enzymes. The only reservation to the experimental results was the fact that no significant decrease in the molecular weight of the human enzyme was observed when compared to the avian prolyl 4-hydroxylase because, under the experimental conditions used here, both enzymes seem to form large molecular weight species (probably protein aggregation) difficult to differentiate from each other. Similarly, no significant decrease in the amino acid (and carbohydrate) composition of the human enzyme was observed, probably due to the use of a more intact (less degradable) enzyme for amino acid and carbohydrate analyses.

The demonstration that the α subunit of the human enzyme was remarkly sensitive to proteolysis may provide insight into the disparate results in previous studies of this enzyme [Nagai and Oka, 1985; Yoshida et al., 1986]. For example, Nagai and Oka [1985] reported that the purified placental prolyl 4-hydroxylase had only β subunits, occurring as 60 kDa monomers or

130 kDa dimers. It is likely that the α subunits in their enzyme preparation were degraded during purification, and that the 130 kDa protein may have been the β-subunit dimer since it had an amino acid composition identical to that of their 60 kDa monomeric subunit. Similarly, studies with monoclonal antibodies directed against human placental prolyl 4-hydroxylase were only able to detect low levels of the α subunit by immunoblotting of the purified human enzyme [Yoshida et al., 1986] consistent with the facile proteolysis of the α subunit. Similarly, Höyhtyä et al. [1984] demonstrated that when holomeric human placental prolyl 4-hydroxylase was used as antigen to obtain monoclonal antibodies, more antibody was produced against the β subunit than the α subunit. More recently, a ratio 1.3 (α) to 4 (β) was reported for purified prolyl 4-hydroxylase from the foot of the marine mussel *Mytilus edulis L.* [Marumo and Waite, 1987]. On the basis of the data reported here, it is suggested that human placental prolyl 4-hydroxylase is found intracellularly as a tetramer composed of two α and two β subunits encoded by two different structural genes, but depending of the amount of protease(s) present in the various tissues that the enzyme have been purified, it is experimentally observed a different molecular composition of the holomeric enzyme when compared with the well-characterized chick embryo holomeric enzyme.

The recent findings that the β subunit of prolyl 4-hydroxylase is essentially identical to the monomeric thyroid binding protein, p55 [Obata et al., 1988], and also to the enzyme protein disulfide isomerase (PDI) [Koivu et al., 1987; Pihlajaniemi et al., 1987; Edman et al., 1985] has attracted much attention and interest [Freedman, 1987, 1989; Lang and Schmid, 1988]. Although the precise role of p55 protein in thyroid function is not known, the synthesis of the p55 transcript is down-regulated by thyroid hormone [Obata et al., 1988]. PDI is involved in the formation of disulfide bonds within and between protein subunits [Freedman 1984; Freedman et al., 1988; Morin and Dixon, 1985]. It appears that the β subunit of prolyl 4-hydroxylase functions as a monomer with p55 thyroid binding activity, as a monomer with PDI activity, and when associated with the α subunit as the heterodimer $\alpha_2\beta_2$, as prolyl 4-hydroxylase. In support of this hypothesis is the fact that the activities of at least seven structurally related proteins (prolyl 4-hydroxylase β subunit, PDI, p55-thyroid hormone binding protein, phosphoinositide-specific phospholipase C, glycosylation site binding protein, iodothyronine-5′-monodeiodinase, and estrogen-binding domain of the estrogen receptor) have been localized to the luminal surfaces of the endoplasmic reticulum [see Freedman, 1989; Kivirikko et al., 1989, 1990; Guzman et al., 1990a].

Thus, the finding that the α subunit of prolyl 4-hydroxylase is extremely labile may be of physiologic importance in regulating the amounts of prolyl 4-hydroxylase, PDI, p55 protein, and the other structurally related proteins in the cell. If the α and β subunits were equally stable, then it is likely that the equilibrium would favor the formation of prolyl 4-hydroxylase. Several studies have reported the relative abundance of β to α subunits in human placenta and in other tissues [Cardinale and Udenfriend, 1974; Prockop et al., 1976; Kivirikko and Myllylä, 1980]. In fact it has long been known that in most tissues inactive β subunits of prolyl 4-hydroxylase are in great excess (fiftyfold in human mammalian liver) of those associated with the holoenzyme [Kivirikko and Myllylä, 1980]. It is likely that there must be a relative abundance of β subunits to α subunits in order to maintain sufficient quantities of PDI, p55, and the other structurally related proteins in the cell. Thus, the relative instability and intracellular degradation of the α subunit may be involved in the regulation of PDI, p55 protein, and the other structurally related protein activities in various tissues. It is notable that the rates of α subunit degradation in adult human spleen and lung is particularly rapid, presumably due to more extensive proteolysis, compared with those of other human tissues [Guzman, unpublished results]. Alternatively, PDI and p55 activity may be controlled by the relative expression of the genes for the α and β subunits and/or the differential sorting of the glycosylated α and non-glycosylated β subunits. Future studies characterizing the amounts of each subunit in different tissues (i.e., the liver and thyroid, etc.) may provide further insight into the factors (e.g., proteolysis, differential synthetic rates, etc.) which regulate the amount of each of these related proteins in various cell and tissue types. Studies of the regulation of the biosynthesis of human prolyl 4-hydroxylase, PDI, p55, and the other structurally related proteins should provide insight into the regulatory mechanism(s) controlling the amounts of these proteins in various tissues and

determine if the in vivo proteolysis of the α subunit is inherently important in the control of all these protein activities in various human tissues.

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