Type IV Collagen from Chicken Muscular Tissues. Isolation and Characterization of the Pepsin-Resistant Fragments†

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ABSTRACT: Type IV collagen has been isolated from adult chicken gizzard after limited pepsin digestion and subsequent differential salt fractionation in acidic and neutral conditions. After denaturation, three fragments (called F1, F2, and F3) were isolated by agarose gel filtration and carboxymethylcellulose chromatography. F1 and F2 possessed apparent molecular weights of 53 000 and 50 000, respectively, and were consistently isolated in a 2:1 proportion. F3 was larger and after reduction of disulfide bonds gave rise to three fragments (called F3A, F3B, and F3C) of apparent molecular weights 68 000, 40 000, and 29 000. No α-chain-sized components of Type IV collagen were observed. A native fraction containing F1 and F2, but no F3, was isolated after extraction using less pepsin and an additional salt fractionation in acidic conditions. F1 and F2 in the native form were not separated by carboxymethylcellulose or diethylaminoethylcellulose chromatography performed in nondenaturating conditions or by differential salt precipitation in acidic or neutral conditions; these results suggest that F1 and F2 arise as a single native component of structure $(F1)_2F2$. The fraction containing F1 and F2 also gave rise to a single segment long spacing crystallite pattern and to a circular dichroism spectrum which was typical for a native collagen. F1 and F2 were also isolated from chicken heart, blood vessels, and skeletal muscle, whereas from bovine aorta, using the same isolation procedures, two α-chain-sized components were obtained, which appeared to be similar to the two Type IV chains recently described by other groups. The data suggest that (i) pepsin fragmentation of Type IV collagen from chicken tissues occurs in a different manner compared to Type IV collagen from mammalian tissues and (ii) for the chicken there must be at least two Type IV chains which are assembled into a single native molecule.

Recently, several investigators have begun to isolate and characterize the collagenous components present in the basement membranes of a variety of tissues. The original analyses of Kefalides and his group [reviewed by Kefalides et al. (1979)] demonstrated that basement membrane collagen is a genetically distinct form of collagen, and it is now usually called Type IV collagen. It was found that after limited pepsin digestion of several basement membranes only a single α-sized chain of Type IV collagen could be isolated (Kefalides, 1971; Dehm & Kefalides, 1978), and it was therefore proposed that Type IV collagen is assembled from three identical chains to give a native molecule of structure $[\alpha 1(IV)]_3$. More recently, however, other groups have proposed that the basement membrane collagenous component is more heterogeneous in composition (Sato & Spiro, 1976; Tryggvason & Kivirikko, 1978), and several groups have now reported the isolation of two α-chain-sized (or slightly smaller) Type IV collagen-like peptides from bovine or human renal glomerulus (Daniels & Chu, 1975; Dixit, 1979), whole human placenta (Glanville et al., 1979; Bailey et al., 1979b; Kresina & Miller, 1979; Sage et al., 1979), and bovine lens capsule (Gay & Miller, 1979; Dixit & Kang, 1979). Several biosynthetic studies have shown that the basement membrane collagen which is synthesized by a variety of cells and tissues in culture can be isolated as a procollagen-like molecule of which each chain has an apparent molecular weight of 160 000–180 000 (Minor et al., 1976; Heathcote et al., 1978; Adamson & Ayers, 1979; Crouch & Bornstein, 1979; Killen & Striker, 1979; Crouch et al., 1980).

Pulse–chase experiments performed with organ cultures of both embryonic rat parietal yolk sac (Minor et al., 1976) and rat lens capsule (Heathcote et al., 1978) have provided evidence that the intact procollagen-like molecule is incorporated directly into basement membrane structure without undergoing any extracellular processing. Additional evidence for the lack of extracellular processing has come from the recent observations that the major collagenous fraction of rabbit kidney tubule basement membrane occurs as multimers of a component of molecular weight 164 000 (Butkowski et al., 1979) and that the basement membrane-like collagen extracted from a transplantable mouse tumor has a molecular weight of approximately 450 000, which is lowered to one-third this value after reduction of disulfide bonds (Timpl et al., 1978). The transplantable mouse tumor may serve as a very useful model system to study the composition of basement membranes as the intact basement membrane procollagen-like molecule can be extracted in large amounts from the tumors of mice made lathyritic by feeding β-aminopropionitrile (Timpl et al., 1978). After pepsin treatment of a solution of extracted tumor collagen, five collagenous peptides have been isolated and characterized, which range in molecular weight from 27 000 to 72 000 (Timpl et al., 1979a,b). Further analyses of these peptides (called P1, P2, P3A, P3B, and P3C) have suggested that the tumor contains at least two different Type IV collagens (Timpl et al., 1979a).

To date, little information is available concerning the structure of Type IV collagen from avian species, and it appears possible that it may differ in several respects from the Type IV collagen of mammals. In this paper we report the isolation and characterization of three pepsin-resistant fragments of Type IV collagen from chicken gizzard, a tissue which is rich in smooth muscle cells. We have called these fragments F1, F2, and F3, and comparison has been made with the fragments which can be isolated from chicken heart, skeletal muscle, and blood vessels and with the inner layers of the tunica media of bovine thoracic aorta. The results show that chicken Type IV collagen differs markedly from bovine aorta

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0006-2960/80/0419-4065$01.00/0

Type IV collagen with regard to the fragments which are generated after limited pepsin digestion and also suggest that chicken Type IV collagen must consist of at least two genetically distinct chains which are assembled into a single native molecule.

Materials and Methods

Tissues. Chicken gizzards either were purchased frozen (Pel-Freez, Rogers, AR) or were dissected from 1-month-old chickens killed in the laboratory. Before extraction the inner and outer connective tissue layers were dissected away from the smooth muscle. Chicken heart (ventricle), arteries (carotid, systemic, and pulmonary), and skeletal muscle (breast) were all dissected from freshly killed chickens. Bovine thoracic aortas were purchased frozen (Pel-Freez), and extraction of collagen was carried out on the inner layer of the tunica media after it had been stripped from both the tunica intima and the outer tunica media plus tunica adventitia.

Isolation of Collagen. All procedures were performed at 4 °C. Chicken gizzards (200 g) were homogenized in 1 M NaCl and 50 mM Tris-HCl, pH 7.5, containing the protease inhibitors $PhCH_2SO_2F$[1] (1 mM), MalNEt (10 mM), and EDTA (20 mM), followed by further extraction with the same solution (48 h, two changes) and with 0.5 M acetic acid (72 h, three changes). The tissue was then stirred for 24 h with 0.5 M acetic acid (2 L) containing pepsin A at various concentrations (1 mg/mL, Sigma; 500 or 100 μg/mL, Worthington), followed by centrifugation (30000*g*, 30 min). The supernatant was adjusted to pH 8.0 by addition with stirring of 5 N NaOH. The pellet of undigested tissue was reextracted for 24 h with the same concentration of pepsin and centrifuged, and this supernatant also was adjusted to pH 8.0. Collagen was precipitated from the combined supernatants by addition of ammonium sulfate (25% saturation) and, after standing overnight, was pelleted by centrifugation (30000*g*, 30 min) and then dissolved in 0.5 M acetic acid (2 L). In some experiments, the solution of collagen in 0.5 M acetic acid was retreated with pepsin (1 mg/mL, Sigma) for 24 h, brought to pH 8.0 with NaOH, and then precipitated again by addition of ammonium sulfate before being dissolved in 0.5 M acetic acid.

Collagen was also extracted from chicken heart, blood vessels, and skeletal muscle by the same procedures as described above after a single extraction in the presence of pepsin at a concentration of 1 mg/mL. For each tissue the volumes of extracting solutions were reduced in proportion to the wet weight of starting material. Bovine aorta tunica media (200 g) was not homogenized prior to extraction but diced into small cubes (1–2 mm^3); otherwise the same isolation procedures were utilized as with the chicken gizzard. Two sequential extractions were carried out in the presence of pepsin (1 mg/mL), but the extracted collagen was not retreated with pepsin.

Fractionation of Collagen Types. Initial separation of Type I and Type III collagens from Type IV and Type V[2] collagens was achieved by differential salt fractionation in acidic conditions as described by Rhodes & Miller (1978). Collagen dissolved in 0.5 M acetic acid (concentration ≃ 1 mg/mL) was dialyzed against 0.7 M NaCl in 0.5 M acetic acid (three changes, 48 h) and the precipitate of Type I and Type III collagen removed by centrifugation. The supernatant was then dialyzed against 1.2 M NaCl in 0.5 M acetic acid (three changes, 48 h) and a precipitate of Type IV and Type V collagen obtained. Type IV collagen was then separated from Type V collagen by differential salt fractionation in neutral conditions. The precipitate of Type IV and Type V collagen was dissolved in 0.5 M acetic acid and dialyzed extensively against 1 M NaCl in 50 mM Tris-HCl, pH 7.5 (four changes, 72 h), followed by dialysis against 2.2 M NaCl, pH 7.5 (three changes, 48 h) which precipitated Type IV collagen components. After removal of the precipitate by centrifugation, Type V collagen was subsequently precipitated from the supernatant by dialysis against 4.4 M NaCl, pH 7.5 (three changes, 48 h). All precipitates of collagen were dissolved in 0.5 M acetic acid, dialyzed extensively against 0.1 M acetic acid, and lyophilized.

Molecular Sieve Chromatography. Initial fractionation of Type IV collagenous components was achieved after denaturation (55 °C, 30 min) by chromatography on a column (2.5 × 155 cm) of agarose beads (Bio-Gel A-5m, 200–400 mesh). The column was eluted with 1 M $CaCl_2$ in 50 mM Tris-HCl, pH 7.5 (Piez, 1968), at a constant flow rate of 16 mL/h. Estimates of apparent molecular weights of basement membrane collagen fragments were obtained by agarose gel chromatography using the same elution conditions as described, except that the column was smaller (1.5 × 155 cm) and the flow rate was 7.5 mL/h. Calibration of all columns was achieved by using a mixture of chicken gizzard Type I and Type III collagens which contained, γ, β, and α components.

CM-cellulose Chromatography (Denaturing Conditions). Samples of collagenous peptides obtained after molecular sieve chromatography were dissolved by warming (45 °C, 30 min) in 0.02 M (Na^+) sodium acetate in 4 M urea (pH 4.8) and applied to a 1.5 × 10 cm column of CM-cellulose (Whatman, CM-32). Elution was achieved with the same buffer and a linear gradient of from 0.0 to 0.12 M NaCl over a total volume of 400 mL. Recovery of collagen after chromatography was always >80%.

Polyacrylamide Gel Electrophoresis. Analysis of protein and peptide fractions was performed by polyacrylamide gel electrophoresis in 50 mM Tris, 30 mM boric acid, pH 8.5, and 0.1% $NaDodSO_4$ as described by Sykes & Bailey (1971). Samples were dissolved in 10 mM Tris in 6 mM boric acid, pH 8.5, containing 0.2% $NaDodSO_4$, 2 M urea, and 10% sucrose, and heated to 100 °C for 1 min either with or without 50 mM dithiothreitol (DTT). Slab gels (140 × 110 × 1.5 mm) were formed with a 3% stacking gel and either a 7.5% or 10% running gel, and separation was achieved with a constant current of 8 mA for 5 h. Gels were stained overnight with 0.1% Comassie brilliant blue (G 250) dissolved in 10% acetic acid (v/v) and 50% methanol (v/v) and destained by diffusion in 7% acetic acid (v/v) and 5% methanol (v/v). Quantitation of bands on stained gels was achieved by using a scanning densitometer (E-C 910, E-C Apparatus Corp., St. Petersburg, FL) at 550–575 nm in the linear range.

Amino Acid Analyses. Lyophilized protein (0.5–1.0 mg) was dissolved in 6 N HCl and hydrolyzed at 105 °C for 20 h in an atmosphere of N_2. Separation of amino acids was achieved with a Beckman 121C amino acid analyzer and the elution program of buffers for a single column as described by Fauconnet & Rochemont (1978).

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[1] Abbreviations used: $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride; MalNEt, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; CM, carboxymethyl; DTT, dithiothreitol; SLS, segment long spacing; DEAE, diethylaminoethyl.

[2] Type V collagen refers to a recently described collagen or family of collagens of which at least three different chains called αA, αB, and αC have now been described. The molecular organization of these chains remains uncertain (Burgeson et al., 1976; Rhodes & Miller, 1978; Bentz et al., 1978; Brown et al., 1978; Sage & Bornstein, 1979; Deyl et al., 1979; Hong et al., 1979).

Peptide Mapping with Submaxillaris Protease. Samples were dissolved in 10 mM Tris in 6 mM boric acid, pH 8.5 (concentration of sample = 2 mg/mL), and incubated at 37 °C with submaxillaris protease (Pierce Chemical Co.) for varying times with a substrate/enzyme ratio by weight of 12.5:1. The reaction was stopped by heating to 100 °C for 1 min, and $NaDodSO_4$ was added to the samples (final concentration = 2%) before analysis by polyacrylamide gel electrophoresis (10% slab gel).

Isolation of F1 and F2 in the Native State. The precipitate of Type IV collagen components obtained after dialysis against 2.2 M NaCl in 50 mM Tris-HCl, pH 7.5, was dissolved in 0.5 M acetic acid at a concentration of 1 mg/mL. This solution was then dialyzed against 0.75 M NaCl in 0.5 M acetic acid, and the precipitate and supernatant were analyzed separately by polyacrylamide gel electrophoresis after desalting and lyophilization.

CM-cellulose Chromatography (Nondenaturing Conditions). Samples were dialyzed against 0.02 M (Na^+) sodium acetate in 4 M urea, pH 4.8 (the starting buffer), before being applied to a 1.5 × 10 cm column of CM-cellulose equilibrated with starting buffer at 15 °C. In some experiments one-half of the sample was denatured by warming to 45 °C for 15 min prior to chromatography. Elution was achieved with a linear gradient of from 0.0 to 0.15 M NaCl over a total volume of 400 mL. Recovery of collagen was estimated by determination of 4-hydroxyproline content before and after chromatography by using an amino acid analyzer.

Circular Dichroism Studies. Spectra were obtained at room temperature with a Cary 61 CD recording spectrophotometer in the wavelength range 190–275 nm. The solution of F1 and F2 (concentration 0.5 mg/mL) isolated after CM-cellulose chromatography in nondenaturing conditions was dissolved in 0.1 M acetic acid. When required, the sample was denaturated by warming to 45 °C for 15 min.

Electron Microscopy. SLS crystallites were prepared by dialysis of collagen solutions (0.5 mg/mL dissolved in 0.1 M acetic acid) for 72 h against 0.4% ATP in 0.1 M acetic acid, pH 2.8 (Timpl et al., 1978). Crystallites were stained with phosphotungstic acid (0.4%, pH 3.5) and uranyl acetate (1%, pH 4.5) and examined in a Philips 200 electron microscope.

DEAE-cellulose Chromatography (Nondenaturing Conditions). The sample was dialyzed against 5 mM Tris-HCl, pH 8.6 containing 2 M urea (the starting buffer) before being applied to a 1.5 × 7.5 cm column of DEAE-cellulose equilibrated with starting buffer at 15 °C. Elution was achieved with a linear gradient of from 0.0 to 0.1 M NaCl over a total volume of 300 mL.

Results

Isolation and Characterization of the Type IV Collagenous Fragments. Initially, experiments were performed with chicken gizzards from which the collagen was solubilized by two sequential extractions in the presence of pepsin (1 mg/mL), and then the extracted collagen was redissolved in 0.5 M acetic acid and treated again with additional pepsin (1 mg/mL). Isolation of the Type IV collagen components from the other collagen types was achieved by differential salt fractionation in acidic and subsequently neutral conditions as described under Materials and Methods. Amino acid analysis of the precipitate obtained at 2.2 M NaCl in 50 mM Tris-HCl, pH 7.5, gave a composition very similar to published values for Type IV collagen. This precipitate was found to weigh from 104.1 to 120.7 mg, this comprising about 6–7% of the total collagen which could be extracted from 200 g of homogenized gizzards. Figure 1 depicts a representative elution

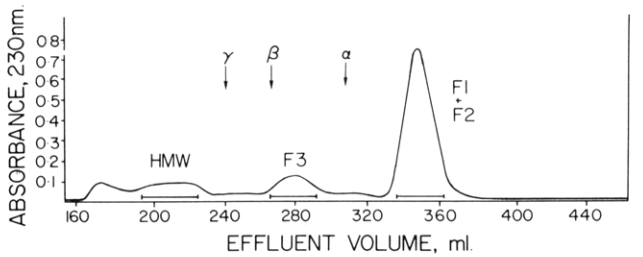

FIGURE 1: Agarose (Bio-Gel A-5m) molecular sieve elution pattern of the Type IV collagen fraction obtained as a precipitate after dialysis against 2.2 M NaCl in 50 mM Tris-HCl, pH 7.5. The column (2.5 × 155 cm) was eluted with 1 M $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, at a constant flow rate, and the sample (20 mg) was denatured in 6 mL of this solution. Arrows show the elution positions of γ (285000), β (190000), and α (95000) components after calibration of the column with a mixture of chicken gizzard Type I and Type III collagens. Bars indicate the fractions which were pooled for further analysis.

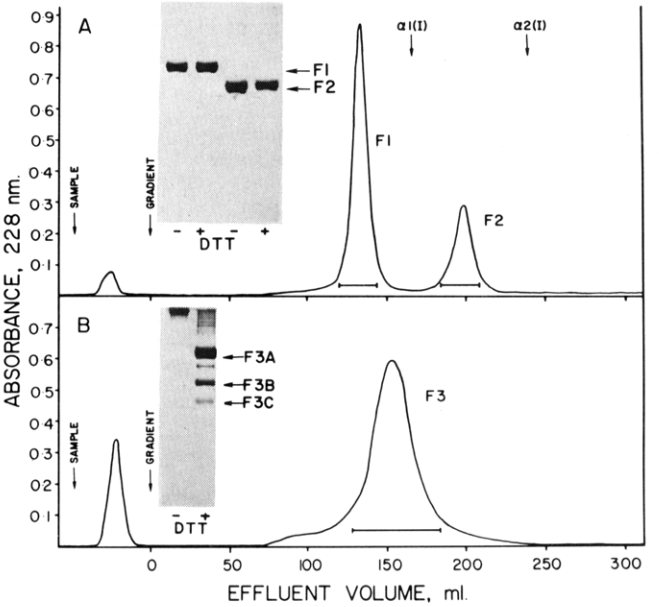

FIGURE 2: CM-cellulose chromatography of the Type IV collagen fragments F1, F2, and F3 after initial separation by agarose gel chromatography (Figure 1). The column (1.5 × 10 cm) was equilibrated at 42 °C with 0.02 M (Na^+) sodium acetate, pH 4.8, containing 4 M urea and elution was achieved with a linear gradient of from 0.0 to 0.12 M NaCl at a flow rate of 100 mL/h over a total volume of 400 mL. Upper panel (A), fractionation of F1 and F2; lower panel (B), fractionation of F3. Arrows show the elution positions of α1(I) and α2 chains present in a preparation of chicken gizzard Type I collagen. Insets show polyacrylamide gel electrophoresis of (A) F1 and F2 and (B) F3 after initial fractionation by CM-cellulose chromatography. Samples were analyzed by polyacrylamide gel electrophoresis (7.5% separating gel) either with or without reduction (50 mM DTT).

pattern of the 2.2 M NaCl precipitate when denatured and chromatographed on a column of agarose beads. Three peaks were resolved during chromatography. A high molecular weight fraction (called HMW) eluted prior to γ, followed by a peak eluting between α and β (called F3) and a third peak eluting after α, which could be further fractionated into F1 and F2 by CM-cellulose chromatography (see below). Each peak was desalted by dialysis against 0.1 M acetic acid and then lyophilized. Further fractionation and purification of F1, F2, and F3 was achieved by chromatography on CM-cellulose (Figure 2). The upper panel (A) shows the separation of the peptides called F1 and F2. F1 was observed to elute before a standard of α1(I) chains while F2 eluted at a location between α1(I) chains and α2 chains. In six separate isolations the absorbance values for F1 and F2 were found to be in a

Table I: Amino Acid Compositions of the Components Recovered after Agarose Gel or CM-cellulose Chromatography[a]

amino acid	residues/1000			
	F1	F2	F3	HMW
3-Hyp	3	<1	3	3
4-Hyp	122	108	140	135
Asp	39	37	51	46
Thr	19	30	26	27
Ser	36	55	27	31
Glu	88	81	95	98
Pro	74	72	66	79
Gly	351	341	302	306
Ala	44	32	33	27
½-Cys	–	–	7	9
Val	26	31	25	23
Met[b]	6	9	11	8
Ile	26	35	20	20
Leu	52	57	64	48
Tyr	10	10	7	2
Phe	18	19	31	23
Hyl	63	38	44	65
Lys	2	3	8	12
His	6	10	6	8
Arg	15	32	34	30

[a] Each analysis is the average of determinations made on three separate preparations and is expressed as residues/1000. F1, F2, and F3 were analyzed after recovery from CM-cellulose chromatography and HMW after agarose gel filtration. No corrections were made for loss of threonine, serine, or cystine or the incomplete release of valine. [d] Determined as the sum of methionine and methionine sulfoxide.

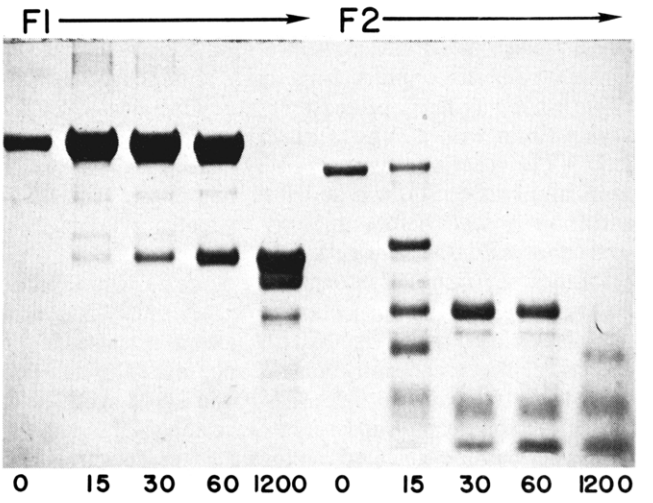

FIGURE 3: $NaDodSO_4$–polyacrylamide gel electrophoresis of F1 and F2 after digestion with submaxillaris protease. The samples (2 mg/mL) were incubated for varying times in 10 mM Tris in 6 mM boric acid, pH 8.5, with a substrate/enzyme ratio by weight of 12.5:1. The reaction was terminated by heating to 100 °C for 2 min, and electrophoresis was carried out on a slab gel (10%) after addition to the sample of $NaDodSO_4$ (final concentration = 2% w/v). Wells were loaded with either 8 μg of protein (no enzyme treatment) or 50 μg of protein (with enzyme treatment).

relatively constant proportion; the mean of the absorbance ratios for F1/F2 was 2.10 (standard deviation ±0.136). This suggested that F1 and F2 might arise as a single native component of molecular composition $(F1)_2F2$ after extraction in the presence of pepsin, and several additional experiments presented later in this paper will provide further evidence supporting this suggestion. The lower panel (B) shows the elution of F3 from CM-cellulose as a single, broad, symmetrical peak close to the location of α1(I) chains. The three fragments F1, F2, and F3 were each desalted by dialysis against 0.1 M acetic acid, lyophilized, and then further analyzed. Figure 2 (upper inset) shows that during polyacrylamide gel electrophoresis the migration of F1 and F2 was unaffected by reduction with DTT. F1 was always observed to migrate more slowly than F2 during electrophoresis. However, the apparent molecular weights of F1 and F2 as determined by molecular sieve chromatography using a calibrated column of agarose beads were 53 000 and 50 000, respectively (data not presented). Figure 2 (lower inset) shows that F3 was unable to migrate into a 7.5% polyacrylamide gel without reduction with DTT. After reduction, three prominent bands were observed which were called F3A, F3B, and F3C. A fourth band which can be observed to migrate between F3A and F3B was not always present. The apparent molecular weights of F3A, F3B, and F3C as determined by molecular sieve chromatography were 68 000, 40 000, and 29 000, respectively (data not presented).

Amino acid compositions of F1, F2, F3, and the high molecular weight fraction called HMW are shown in Table I. F1, F2, and F3 were obtained after fractionation by CM-cellulose chromatography (Figure 2), and HMW was obtained after agarose gel chromatography (Figure 1). In general, the results showed compositions for all four fractions similar to those reported for Type IV collagen components isolated from other species and tissues. Typically, our fractions showed a low alanine content, a high imino acid content with an excess of 4-hydroxyproline over proline, and the almost complete hydroxylation of lysine residues. Amino acid compositions of F1 and F2 consistently showed differences in the contents of 4-hydroxyproline, threonine, serine, hydroxylysine, and arginine. F3 was different from F1 and F2, having less than one-third glycine and also containing cysteine; these results suggest that F3 probably contains at least one noncollagenous domain. The fraction HMW showed similarities to F3 in amino acid composition, and after reduction HMW gave rise to three prominent bands which migrated on $NaDodSO_4$–polyacrylamide gel electrophoresis to the same extent as F3A, F3B, and F3C (data not presented). HMW may therefore exist in part as partially cleaved "aggregates" of F3 which are maintained to each other or to other proteins by disulfide bonds.

The amino acid analyses of F1 and F2 showed that F2 has approximately twice the arginine content of F1, and this result suggested that these two fragments might differ in their susceptibility to cleavage by mouse submaxillaris protease, an enzyme considered specific for arginine residues (Schenkein et al., 1977). Figure 3 shows that F1 was poorly cleaved by this protease, with appreciable cleavage occurring only after 1200 min of incubation. In contrast, F2 was extensively cleaved after only 15 min of incubation, and by 1200 min was almost completely cleaved to small fragments which were no longer retained in the gel after staining and destaining.

Effects of Varying the Pepsin Concentration and Extent of Pepsin Digestion on the Isolation of Type IV Collagenous Components. Experiments were next performed in which gizzards were twice extracted with pepsin at concentrations of 100 or 500 μg/mL for 24 h at 4 °C, and the isolated collagen was not retreated with additional pepsin before being subjected to differential salt fractionation as described in previous experiments. It was found that F3 was not extracted in significant amounts during these experiments and that the yields of F1 and F2 were markedly reduced when pepsin was present at a concentration of only 100 μg/mL. At no time with any of the pepsin concentrations used did we observe any Type IV like α-size components; this contrasts with previous

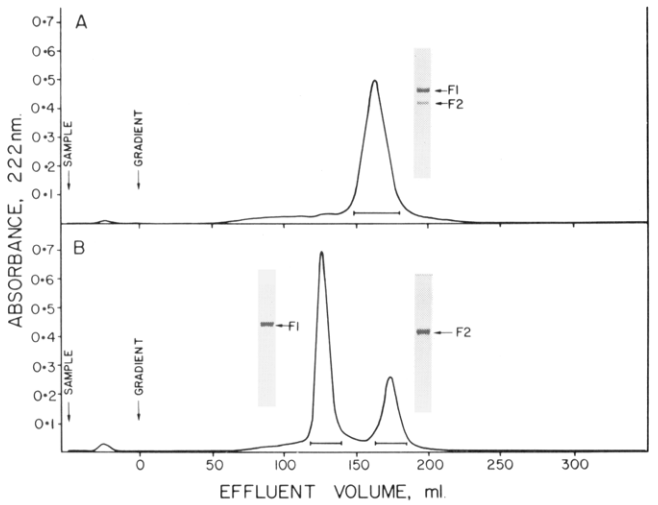

FIGURE 4: CM-cellulose chromatography (nondenaturing conditions) of F1 and F2 isolated in the native form after additional differential salt fractionation in acidic conditions. The column (1.5 × 10 cm) was equilibrated at 15 °C with 0.02 M (Na^+) sodium acetate, pH 4.8, containing 4 M urea, and elution was achieved with a linear gradient of from 0.0 to 0.15 M NaCl at a flow rate of 100 mL/h over a total volume of 400 mL. Prior to chromatography, one-half of the sample was denatured by warming to 45 °C for 15 min. Upper panel (A): fractionation of F1 and F2 without prior denaturation; recovery of hydroxyproline was 57%. Lower panel (B): fractionation of F1 and F2 after denaturation; recovery of hydroxyproline was 74%. Bars indicate fractions which were pooled for further analysis. Insets show polyacrylamide gel electrophoresis (7.5% separating gel) of pooled fractions after desalting and lyophilization.

results with human and bovine tissues (Daniels & Chu, 1975; Dixit, 1979; Glanville et al., 1979; Bailey et al., 1979b; Kresina & Miller, 1979; Sage et al., 1979; Gay & Miller, 1979; Dixit & Kang, 1979) and with our own results with bovine aorta (see below).

Isolation and Characterization of a Native Fraction Containing Only F1 and F2. In earlier experiments (e.g., Figure 2) we found that the absorbance for F1 was consistently twice that of F2 after separation by CM-cellulose chromatography. Moreover, a constant proportion of F1 to F2 was always observed despite widely varying yields of these components as a result of solubilization in the presence of different pepsin concentrations. This suggested that F1 and F2 might arise as a native component of structure $(F1)_2F2$, and further experiments were performed to attempt to isolate and characterize such a component. The precipitate of Type IV collagen components which was obtained after two extractions in the presence of pepsin (500 μg/mL) and subsequent fractionation by differential salt precipitation at 2.2 M NaCl, pH 7.5, was redissolved in 0.5 M acetic acid, and the concentration of collagen was adjusted to 1.0 mg/mL. This solution was then dialyzed extensively against 0.75 M NaCl in 0.5 M acetic acid and the collagen present in both the precipitate and the supernatant analyzed by polyacrylamide gel electrophoresis. The supernatant contained only F1 and F2, whereas the precipitate contained, in addition to some F1 and F2, several high molecular weight components which only appeared after reduction (data not presented).

Further purification of the native form of F1 and F2 was achieved by CM-cellulose chromatography performed in nondenaturing conditions. The supernatant containing F1 and F2 obtained after differential salt fractionation at 0.75 M NaCl in 0.5 M acetic acid was dialyzed extensively against starting buffer for CM-cellulose chromatography. Figure 4A shows the results of chromatography performed on one-half of the sample with the column maintained at 15 °C. A single, sharp, symmetrical peak was observed which was shown to contain both F1 and F2 after desalting and $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 4A, inset). The remaining half of the sample was first denatured by warming to 45 °C for 15 min prior to chromatography, and then chromatography was performed at 15 °C (Figure 4B). Separation of F1 and F2 now occurred with the absorbance for F1 being twice that for F2.

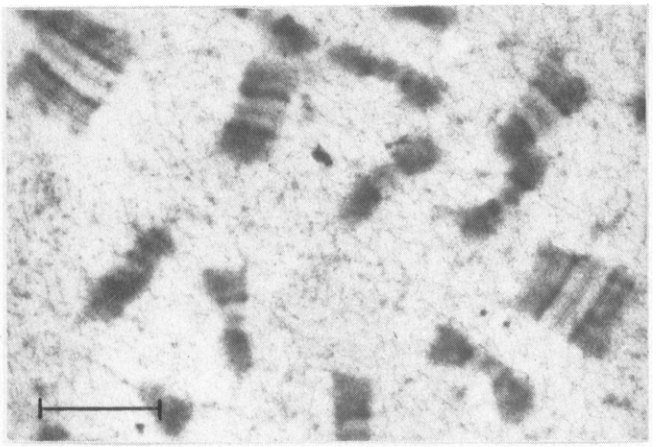

FIGURE 5: Electron micrograph of SLS crystallites prepared from a native preparation of F1 and F2 isolated after CM-cellulose chromatography in nondenaturing conditions (Figure 4A). Only a single crystallite pattern was observed, consisting of a dimer of two crystallites joined end-to-end to each other in a mirror plane. The bar represents the length of a crystallite of type I collagen (290 nm) after examination on the same grid as a preparation of F1 and F2. Staining was with phosphotungstic acid (0.4%, pH 3.5) and uranyl acetate (1%, pH 4.5).

Several additional experiments were performed with F1 and F2 isolated after CM-cellulose chromatography in nondenaturing conditions (Figure 4A). Dialysis of the fraction containing F1 and F2 against either 1.2 M NaCl in 0.5 M acetic acid or 2.2 M NaCl in 50 mM Tris-HCl, pH 7.5, resulted in the precipitation of both F1 and F2 as shown after analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. No precipitation of F1 and F2 occurred if these fragments were first denatured prior to CM-cellulose chromatography (Figure 4B) and subsequently dialyzed against 1.2 M NaCl in 0.5 M acetic acid or 2.2 M NaCl, pH 7.5. Precipitation of F1 and F2 in conditions known to cause the precipitation of other native collagens suggests that these fragments are present in a triple helical conformation, and this was confirmed by comparison of the circular dichroism spectrum for F1 and F2 with that of known collagens. The circular dichroism spectrum for F1 and F2 isolated as shown in Figure 4A gave a small positive peak at 222 nm and a large negative peak at 198 nm. After denaturation, the positive peak was no longer present and the negative peak was markedly reduced in magnitude (data not presented). The circular dichroism spectrum for F1 and F2 isolated in nondenaturing conditions and the changes in the spectrum after denaturation were both very similar to those obtained for interstitial and basement membrane collagens (Hayashi et al., 1979; Timpl et al., 1978).

SLS crystallites of a solution containing F1 and F2 were prepared by dialysis against an acidic solution of ATP. Analysis of the precipitate and supernatant by $NaDodSO_4$–polyacrylamide gel electrophoresis and subsequent scanning of stained gels showed that by 72 h 70–80% of the total F1 and F2 was precipitated. When the crystallites were examined in the electron microscope, only a single crystallite pattern was observed (Figure 5), the pattern representing a dimer of two crystallites joined end to end to each other in a mirror plane.

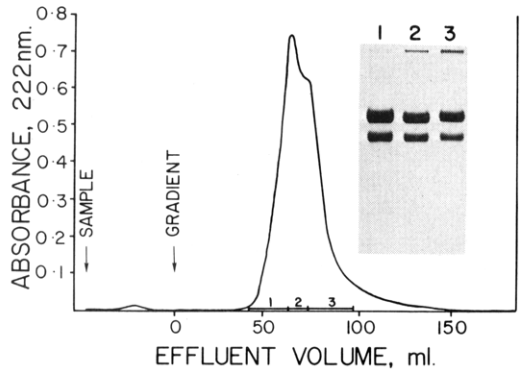

FIGURE 6: DEAE-cellulose chromatography (nondenaturing conditions) of F1 and F2 after isolation by an additional differential salt precipitation. The column (1.5 × 7.5 cm) was equilibrated at 15 °C with 5 mM Tris-HCl, pH 8.6, containing 2 M urea, and elution was achieved with a linear gradient of from 0.0 to 0.1 M NaCl at a flow rate of 100 mL/h over a total volume of 300 mL. Recovery of hydroxyproline was 92%. Three fractions of the peak (designated 1, 2, and 3) were desalted, lyophilized, and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (inset). Densometric scanning of the gels showed that for all three fractions the absorbance for F1 was twice that for F2.

The length of a single crystallite of F1 and F2 was determined to be 130–150 nm by comparison with SLS crystallites prepared from Type I collagen and examined on the same grid.

Experiments were also performed in an attempt to fractionate F1 and F2 in the native state by DEAE-cellulose chromatography in nondenaturing conditions as described by Timpl et al. (1979a). Figure 6 shows that F1 and F2 eluted as a single peak during DEAE-cellulose chromatography performed at 15 °C. The three areas of the peak (shown as 1, 2, and 3) were each desalted and lyophilized. The inset shows $NaDodSO_4$–polyacrylamide gel electrophoresis performed on these fractions. All three fractions were found to contain F1 and F2, and densometric scanning of the gels gave F1/F2 absorbance ratios for the fractions 1, 2, and 3 of 2.33, 2.05, and 2.15, respectively.

Attempts were also made to separate the native form of F1 and F2 by differential salt fractionation in either acidic or neutral conditions. In acidic conditions the sample of F1 and F2 was dialyzed against increasing concentrations of salt dissolved in 0.5 M acetic acid. Precipitations were observed at 0.85 M NaCl and 0.95 M NaCl, which were centrifuged out, redissolved in acetic acid, desalted by dialysis, and lyophilized. Analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis and subsequent densometric scanning of the gels after staining gave F1/F2 absorbance ratios for the 0.85 M NaCl and 0.95 M NaCl precipitates of 2.37 and 2.49, respectively. Similar experiments were performed in neutral conditions where precipitates were observed at 1.5, 1.7, and 1.9 M NaCl in 50 mM Tris-HCl, pH 7.5. F1/F2 absorbance ratios for these precipitates were determined to be 2.34, 2.38, and 2.48, respectively. In subsequent experiments it was realized that the F1/F2 absorbance ratio will increase with longer destaining times, and the overnight destaining carried out in this series of experiments probably explains the slight elevation of the absorbance ratios above 2.

Additional experiments were performed to compare the pepsin-resistant fragments of Type IV collagen isolated from chicken gizzard with those isolated from other chicken muscular tissues and from the inner layer of the tunica media of bovine aorta. The isolation and fractionation methods were the same for all tissues except that the chick tissues were extracted only once in the presence of pepsin (1 mg/mL) whereas the bovine aorta was extracted twice. Analyses by polyacrylamide gel electrophoresis of the collagens precipitated

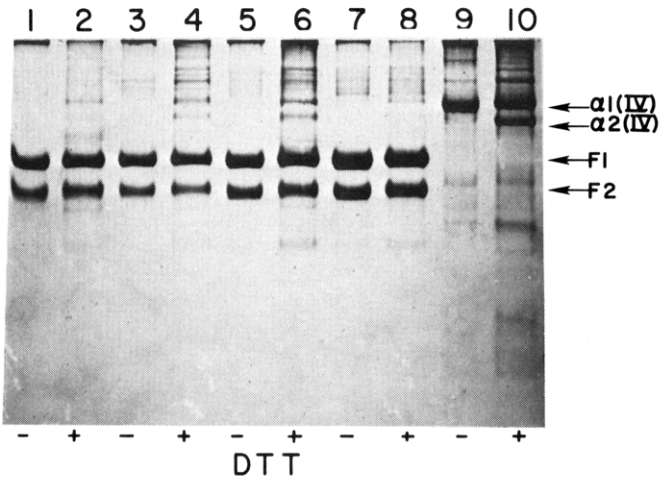

FIGURE 7: $NaDodSO_4$–polyacrylamide gel electrophoresis (7.5% separating gel) of the Type IV collagenous peptides isolated from several tissues after differential salt fractionation of the precipitate obtained by dialysis against 2.2 M NaCl in 50 mM Tris-HCl, pH 7.5. The tissues analyzed were (1 and 2) chicken blood vessels, (3 and 4) chicken cardiac muscle, (5 and 6) chicken striated muscle, (7 and 8) chicken gizzard, and (9 and 10) bovine aorta. Samples were analyzed either with or without reduction (50 mM DTT). F1 and F2 were found to be the major collagenous components for all chicken muscular tissues analyzed, whereas for the bovine aorta two α-sized components were obtained, one of which migrated more rapidly after reduction. The two α-sized components were labeled α1(IV) and α2(IV) following the designation of Crouch et al. (1980) and are equivalent to the C and D components of Kresina & Miller (1979).

at 2.2 M NaCl in 50 mM Tris-HCl, pH 7.5, are shown in Figure 7. F1 and F2, or similar peptides, were isolated from all chicken tissues (blood vessels, cardiac muscle, striated muscle, and gizzard), whereas from bovine aorta F1 and F2 were not present in detectable amounts and instead a prominent α-sized band was observed which after reduction separated into two bands, one of α size and the other of apparent molecular weight 80 000. These components have now been isolated and characterized (Mayne, Zettergren, Mayne, and Bedwell, unpublished observations) and appear to be similar, if not identical, to the two type IV chains recently described in other tissues (Glanville et al., 1979; Kresina & Miller, 1979; Sage et al., 1979; Dixit, 1979; Gay & Miller, 1979; Dixit & Kang, 1979).

Discussion

From our results it would appear that the fragments of Type IV collagen which can be isolated from chicken muscular tissues after limited pepsin digestion differ from the fragments which other groups have isolated from mammalian tissues. This is particularly evident by our inability to isolate any α-sized components from any of the chicken tissues investigated, despite the use of protease inhibitors and a wide range of pepsin treatments. It appears likely that this difference in fragmentation by pepsin is due to a difference in species, particularly with regard to the location of the pepsin-sensitive sites within the molecule. This is shown most clearly by the data presented in Figure 7. The fragments F1 and F2 have been obtained from all chicken tissues including chicken blood vessels, whereas from bovine aorta no F1 and F2 can be detected and instead two α-size components are prominent which appear to be the same as the two Type IV like α-sized components described by other groups. This apparent difference in sensitivity of chicken Type IV collagen to pepsin may explain the failure of Trelstad (1979) to isolate a typical Type IV collagen from chicken embryos and may also explain why Bailey et al. (1979a) failed to find any Type IV collagen in chicken skeletal muscle.

During the isolation of Type IV collagen from most mammalian tissues, in addition to α-sized and higher molecular weight components, substantial quantities of fragments of molecular weight $\simeq$50 000 are often observed (Dixit, 1979; Dixit & Kang, 1979; Glanville et al., 1979; Kresina & Miller, 1979; Gay & Miller, 1979). It is generally assumed that these fragments arise as the result of an additional pepsin cleavage within a triple helical domain. This viewpoint is supported by the work of Schwartz & Veis (1978), who have prepared SLS crystallites from pepsin-digested lens capsule and have observed crystallites which were both 300 nm (α sized) and 135 nm in length. The cross-striations of the 135-nm segment were found to correspond with one end of the 300-nm segment. In other studies in which the lower molecular weight fragments were isolated from whole placenta (Kresina & Miller, 1979) or lens capsule (Gay & Miller, 1979), two major classes of fragments of apparent molecular weight $\simeq$50 000 were fractionated which from their amino acid composition and chromatographic behavior on CM-cellulose were thought to originate from additional proteolytic cleavages of two Type IV chains called C and D. The fragments F1 and F2 show some similarities both in amino acid composition and molecular weight to the lower molecular weight fragments isolated by this group, and it is possible that they also originate from additional pepsin cleavage sites within an α-sized domain of the molecule. However, such sites, if they exist for the chick, must be very easily cleaved by pepsin so that significant amounts of α-sized components are never formed.

The initial observation that F1 and F2 were consistently isolated in a 2:1 proportion after denaturation and CM-cellulose chromatography (Figure 2) suggested that F1 and F2 might be assembled as a single native component. We have found that it is possible to isolate the native form of F1 and F2 from all other contaminating components by additional differential salt fractionation. The observations that F1 and F2 in the native state cannot be separated by CM-cellulose or DEAE-cellulose chromatography performed in nondenaturing conditions nor by differential salt precipitation in neutral or acidic conditions all suggest that F1 and F2 arise as a single native component of structure $(F1)_2F2$. The observation of a single segment long spacing crystallite which is approximately one-half of the length of a segment long spacing crystallite of Type I collagen also supports this suggestion. Little definitive information is at present available concerning the molecular organization of the two Type IV collagen components which have been isolated from mammalian tissues, although Timpl et al. (1979a) have concluded that the transplantable mouse tumor contains two different Type IV molecules and Gay & Miller (1979) have suggested that bovine lens capsule may also contain two Type IV molecules.

The three fragments F1, F2, and F3 show many similarities in size, in chromatographic properties in various systems, and in amino acid composition to the three fragments called P1, P2, and P3 which have been obtained after pepsin treatment of the basement membrane collagen extracted from the transplantable mouse tumor (Timpl et al., 1979a,b). This is especially evident when the fragments F3 and P3 are compared. Both are very similar in amino acid composition and also give rise to three fragments of very similar size after reduction of disulfide bonds. Another similarity between the Type IV collagen of the transplantable tumor and chicken Type IV collagen is that neither gives rise to significant amounts of α-sized components after pepsin treatment. However, our results differ from those of Timpl et al. (1979a) in one important respect. We have evidence that F1 and F2 probably occur as a single triple helical component of structure $(F1)_2F2$, whereas Timpl et al. (1979a) have found that the native forms of P1 and P2 can be separated on DEAE-cellulose chromatography carried out in nondenaturing conditions and that therefore P1 and P2 probably occur as two different components of structure $(P1)_3$ and $(P2)_3$. We have attempted to separate the native form of F1 and F2 by DEAE-cellulose chromatography in nondenaturing conditions as described by Timpl et al. (1979a) but failed to achieve any separation (Figure 6).

At present the structure of F3 is still uncertain, and we have been unable to isolate F3 in the native state. It is also unclear whether F3 is a component of the same native molecule as F1 and F2. We have, however, consistently observed that the absorbance for F3A is approximately twice the sum of the absorbances for F3B and F3C, and so a possible structure for F3 might be $(F3A)_2(F3B + F3C)$, such a structure being maintained by disulfide bonds. Additional evidence supporting such a structure comes from our estimates of the apparent molecular weights for F3A, F3B, and F3C, the molecular weight for F3A (68 000) being very close to the sum of the molecular weights for F3B (40 000) and F3C (29 000). A model can therefore be devised for the structure of F3 which is compatible with F3 being a separate domain of the same molecule as F1 and F2. However, at present, our investigations have only been concerned with those domains of chicken Type IV collagen which are resistant to pepsin digestion, and further additional experiments are now required to isolate and characterize the intact molecule and to determine the possible location of F1, F2, and F3 within such a molecule.

Acknowledgments

We thank Noel Bedwell, Pauline Mayne, and Lezlie Carroll for their excellent technical assistance during this work, John Ford for performing the amino acid analyses, Frank Winfrey for photography and the electron microscopic observations, and Dr. Marianna Long for the circular dichroism spectra.

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Studies on Human Lactoferrin by Electron Paramagnetic Resonance, Fluorescence, and Resonance Raman Spectroscopy†

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ABSTRACT: Investigations of metal-substituted human lactoferrins by fluorescence, resonance Raman, and electron paramagnetic resonance (EPR) spectroscopy confirm the close similarity between lactoferrin and serum transferrin. As in the case of Fe(III)- and Cu(II)-transferrin, a significant quenching of apolactoferrin's intrinsic fluorescence is caused by the interaction of Fe(III), Cu(II), Cr(III), Mn(III), and Co(III) with specific metal binding sites. Laser excitation of these same metal-lactoferrins produces resonance Raman spectral features at ca. 1605, 1505, 1275, and 1175 cm^{-1}. These bands are characteristic of tyrosinate coordination to the metal ions as has been observed previously for serum transferrins and permit the principal absorption band (λ_{max} between 400 and 465 nm) in each of the metal-lactoferrins to be assigned to charge transfer between the metal ion and tyrosinate ligands. Furthermore, as in serum transferrin the two metal binding sites in lactoferrin can be distinguished by EPR spectroscopy, particularly with the Cr(III)-substituted protein. Only one of the two sites in lactoferrin allows displacement of Cr(III) by Fe(III). Lactoferrin is known to differ from serum transferrin in its enhanced affinity for iron. This is supported by kinetic studies which show that the rate of uptake of Fe(III) from Fe(III)–citrate is 10 times faster for apolactoferrin than for apotransferrin. Furthermore, the more pronounced conformational change which occurs upon metal binding to lactoferrin is corroborated by the production of additional EPR-detectable Cu(II) binding sites in Mn(III)–lactoferrin. The lower pH required for iron removal from lactoferrin causes some permanent change in the protein as judged by altered rates of Fe(III) uptake and altered EPR spectra in the presence of Cu(II). Thus, the common method of producing apolactoferrin by extensive dialysis against citric acid (pH 2) appears to have an adverse effect on the protein.

Human lactoferrin is one of the class of nonheme, iron-binding proteins generally designated as the transferrins. However, because the importance of lactoferrin in human physiology has only recently become apparent, it has not been as extensively studied as the other members of the class, serum transferrin and ovotransferrin. Lactoferrin occurs in high concentration in human milk and has been found in a variety of other bodily secretions and in intracellular components (Aisen, 1973; Bezkorovainy, 1977; Feeney & Komatsu, 1966). Because of its avidity for Fe(III), it has been postulated to have a bacteriostatic function in depriving microorganisms of essential iron required for their growth (Bullen et al., 1974). A nutritional role for human lactoferrin as an iron (McMillan et al., 1976) as well as a zinc carrier (Ainscough et al., 1980) has also been suggested.

All the transferrins have a molecular weight of ~80 000 and are capable of binding specifically two Fe(III) ions in

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0006-2960/80/0419-4072$01.00/0