Intestinal Basement Membrane of *Ascaris suum*

MOLECULAR ORGANIZATION AND PROPERTIES OF THE COLLAGEN MOLECULES*

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The collagenous components of *Ascaris suum* intestinal basement membrane were isolated by extraction with 0.1 M Tris-HCl, 0.5 M NaCl, 0.5% 2-mercaptoethanol, pH 8.3, and Sephacryl S-300 gel filtration. Rotary-shadowing electron microscopy showed that the collagenous components occur as monomers and dimers with mean contour lengths of 469 ± 21 and 918 ± 24 nm, respectively. The molecules each contain a globular domain, with that of the dimer being slightly larger than that of the monomer. Electrophoresis in sodium dodecyl sulfate-polyacrylamide gels under reducing conditions revealed two polypeptides of Mr = 185,000 and 179,000. A similarity to type IV collagen was noted, with a major cross-linking site that is analogous to that found in basement membranes of mammalian counterparts yet has a less complicated structure. Hence, it has been studied to elucidate fundamental structure features of basement membranes. Structural studies revealed that the major collagenous constituent, excised by pepsin digestion, is organized in the form of two triple-helical molecules held together in an end-to-end fashion through disulfide bonds. This dimeric molecule was envisioned to be a fundamental structural unit which was derived from a larger precursor by the action of pepsin (2).

More recent studies of mammalian basement membrane have demonstrated that basement membrane collagen exists in the form of a triple-helical molecule with a globular domain at the carboxyl-terminal end. This domain is a major cross-linking site in the supramolecular assembly of the collagen in the membrane matrix (3–5). A second major cross-linking site has been identified at the amino-terminal end (3, 6). Presumably, the dimeric unit described for *Ascaris* basement membrane represents a remnant of dimeric molecules arising from cross-links at either the amino- or carboxyl-terminal end of a monomer.

The purpose of this study was to define the molecular organization and properties of the assembly of collagenous molecules from which the dimeric collagenous component was derived. To accomplish this, a chemical solubilization procedure was developed which preserved the triple-helical conformation of the collagenous molecules. The present study demonstrated the existence of a collagen molecule that has a globular domain at one end. Two of these form a dimer through interaction of their globular domains, which reflects a major cross-linking site that is analogous to that found in mammalian basement membranes.

**EXPERIMENTAL PROCEDURES**

**Materials**

All electrophoretic chemicals were purchased from Bio-Rad, Sephacryl S-300 from Pharmacia, and 2-mercaptoethanol from Eastman. Urea solutions (Baker) were filtered and deionized prior to use. Protease inhibitors (disopropylphosphorofluoridate, N-ethylmaleimide, sodium-EDTA, and 6-aminocaproic acid) which were employed for the isolation of *Ascaris* membrane were obtained from Sigma. Pepsin was from Worthington and bacterial collagenase from Advanced Bio-Factors. All other reagents were the best commercially available and were used without further purification.

**Methods**

Preparation of *A. suum* Intestinal Basement Membrane—*Ascaris* membrane was isolated by the procedures described previously (7).

Isolation of the Collagenous Constituents of *A. suum* Intestinal Basement Membrane—Thiol-solubilized Collagen—*A. suum* intestinal basement membrane was suspended at a concentration of 1 mg/ml in 0.1 M acetic acid and stirred at room temperature for 24 h. The pellet, collected by centrifugation at 10,000 × g for 15 min, was suspended in 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.3, at approximately 1 mg/ml and stirred overnight at room temperature. The mixture was centrifuged and the pellet was suspended at 1 mg/ml in 0.1 M Tris-HCl, 0.5 M NaCl, 0.5% 2-mercaptoethanol (v/v), pH 8.3, and stirred at room temperature for 24 h and centrifuged. The supernatant fluid, containing the collagenous constituents of *Ascaris* membrane, was alkylated in the above buffer with a 2–3-fold molar excess of sodium ioodeacetate or iodoacetamide over 2-mercaptoethanol for 2 h. Ten ml of this solution was then applied to a Sephacryl S-300 column (2 × 90 cm), equilibrated in 0.1 M Tris-HCl, 0.5 M NaCl, 0.5% 2-mercaptoethanol (v/v), pH 8.3, and eluted at a flow rate of 12 ml/h. Fractions of 2.5 ml were collected and their relative protein concentrations were determined by absorbance measurements at 230 nm.

**Pepsin-solubilized Collagen**—This was isolated as described previ-

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Electron Microscopy—Conditions used for rotary shadowing electron microscopy were previously described (6). All samples were sprayed at acid pH (0.025 M acetic acid, 50% glycerol, pH 3.5) with the exception of bacterial collagenase-resistant products which were sprayed at both neutral pH (0.075 M ammonium bicarbonate, 50% glycerol, pH 7.5). Contour lengths, end-to-end measurements, and diameters were determined from photographs, at a total magnification of 240,000, using a Hipad digitizer interfaced to a Digital computer. The error in measurement of contour length and end-to-end distance was 1.7% as determined by 25 measurements on a single molecule.

Electrophoresis—Electrophoresis was performed employing four different systems as previously described (8). In system I, electrophoresis was performed on 5% polyacrylamide gels in 0.1% SDS, 0.1 M sodium phosphate at pH 7. System II was identical to system I with the exception that 8 M urea was included in the gel. System II consisted of 3% agarose gels in 0.1% SDS, 0.1 M sodium phosphate at pH 7. System IV employed 12% discontinuous polyacrylamide gels in SDS as described previously (9). Gels were stained in 5% Coomassie Brilliant Blue, 10% acetic acid, and 40% isopropanol for 6 h and then dried in a diffusion chamber containing 10% acetic acid. Gels were scanned at 650 nm employing a Gilford spectrophotometer with a scanning accessory.

Molecular weight estimates were obtained from 5 or 12% polyacrylamide and 3% agarose gels employing multimers of type I collagen as standards (6). In the 3% agarose gels, the migration position of 12 multimeric units of the α chain could be identified by varying the full scale absorbance on a gel scan, thus providing a calibration line from molecular weight 95,000–1,140,000.

Chemical Analysis—Samples for amino acid analysis were hydrolyzed in 6 N constant-boiling HCl under reduced pressure in sealed tubes at 105 °C for 24 h. The composition was determined on a Beckman 121 HP amino acid analyzer employing a dual column method (Beckman manual). Samples for amino sugar analysis were hydrolyzed under identical conditions for 4 h with 4 N HCl. The hydrolysates were analyzed on the short column of the analyzer.

Enzymatic Digestions—Thiol-solubilized collagenous constituents extracted from Ascaris membrane were dissolved at a concentration of 5 mg/ml in 0.1 M acetic acid at 4 °C, and then pepsin was added to give a 1:10 pepsin to substrate ratio. Digestion was allowed to proceed for 24 h at 4 °C, at which time the digestion mixture was made 0.1 M in Tris-Cl and neutralized with sodium hydroxide. The remaining collagenous domains were precipitated with an equal volume of cold 5 M NaCl, followed by a second suspension and precipitation step. An unfractionated digest was used for electron microscopy studies.

Thiol-solubilized Ascaris collagen was suspended at a concentration of 2 mg/ml in 0.025 M Tris-Cl, 0.01 M calcium acetate, pH 7.4, under a layer of toluene. Approximately 125 units of bacterial collagenase was added for each 2 mg. The digestion was allowed to proceed at 37 °C for 20 h with stirring. Under the conditions described, 2 mg of type I collagen were completely degraded in less than 4 h as evaluated by SDS-PAGE. The reaction was terminated by addition of stock Na₂EDTA to obtain a final concentration of 20 mM. Samples were dialyzed against dilute acetic acid and lyophilized.

RESULTS

In a previous study, we showed that the major component of pepsin-solubilized collagen from A. suum intestinal basement membrane collagen exists in the form of a dimer composed of two triple-helical units which are joined end-to-end by disulfide bonds (2). Because we found that the dimer dissociated into its monomeric units upon selective reduction of disulfide bonds under conditions which did not unfold the triple helix, we attempted to solubilize collagen from the whole membrane by use of this thiol-reductive procedure. The aim was to obtain a molecule which had not been proteolytically degraded and was thus more representative of collagen in the intact membrane. We obtained such a molecule, but, in addition, obtained a dimer of it in which two globular domains, each located at an end of a monomer, interacted. Thiol-solubilized collagen was obtained in high yield (70%) as was the pepsin-solubilized dimer (90%) that we have previously described (8). This indicates that both are derived from the same supramolecular assembly of collagen molecules in the basement membrane and, consequently, must have large regions in common. The results of the characterization of the thiol-solubilized collagen and the comparison of it to pepsin-solubilized collagen are presented below.

Characterization of Thiol-solubilized Collagen—Using conditions similar to those previously documented by circular dichroism to preserve triple-helical conformation (2), A. suum intestinal basement membrane collagen was solubilized by partial reduction of disulfide bonds (Fig. 1). Eighteen per cent of the membrane was solubilized, as based on dry weight measurements; this represents approximately 70% of the membrane collagen (8). SDS-PAGE of the solubilized components after reduction revealed principally two bands (Fig. 1) which had been identified earlier as the collagenous constituents of Ascaris membrane (7). The collagenous components were fractionated from lower M proteins by gel filtration through a column of Sephacryl S-300 under nondenaturing conditions (Fig. 2).

Rotary-shadowing electron microscopy was performed on various fractions from the S-300 column. Fractions from the leading edge of the major peak contained molecules (Fig. 3, A–D) similar in size and shape to monomer and dimer forms of type IV collagen seen in other systems (3, 10–13). Characteristic dimensions of Ascaris collagen molecules are presented in Table I. The monomer-sized component of thiol-solubilized collagen was determined to have a mean contour length of 469 ± 21 nm (24 molecules) and to end in a globular domain with a mean diameter of 17 ± 2 nm. The length is almost identical to that of collagen from Drosophila basement membrane (12) but slightly larger than that from mice (3, 10, 11). The dimer-sized component (Fig. 3, C and D) has a mean contour length of 918 ± 24 nm (25 molecules) and has a globular domain at its center which has a mean diameter of 23 ± 2 nm. Thus, the dimer-sized component consists of two monomers joined through their globular domains. The Ascaris thiol-solubilized dimer has a gross configuration which is quite similar to that of a type IV collagen dimer which was extracted from a lathyritic Engelbreth-Holm-Swarm mouse tumor with 2 M guanidine HCl, 1 mM dithiothreitol (15). The Ascaris collagen dimer is slightly longer than the mouse collagen dimer. Deposition of platinum onto the surface of the collagen molecule causes an inherent error when measuring either the length of the molecule or the diameter of the globular domain. The diameter of the platinum crystallites is on the order of 1–2 nm and thus will affect the actual diameter of the globular domains to a greater extent than the lengths of the molecules.

Approximate molecular weights were calculated from the lengths of the triple-helical domains (i.e. contour length minus the diameter of the noncollagenous globular domain); the length/residue of 0.296 nm for a single collagen chain in triple-helical conformation (14); a mean residue weight of 99 and a carbohydrate content of 12.5% (w/w), which were calculated from the chemical composition (Table II); and the molecular weight of the noncollagenous domain (30,000/chain, discussed below). The calculated molecular weights are 625,000 and 1,284,000 for the monomer and dimer, respectively.

SDS-agarose gels (Fig. 4, gel B) support the existence of both monomeric and dimeric collagen since the mobilities of the upper and lower bands indicate a molecular weight ratio.
Ascari
collagen affect the electrophoretic properties of the
monomer and dimer in much the same way. This, in addition
to the dimer being a linear combination of monomers, could
cause Ascari
collagen to have a log $M$, versus mobility curve parallel to one based on linear type I collagen multimers and thus yield a correct ratio of monomer to dimer mobilities.

Pooled fractions from S-300, when evaluated by SDS-
PAGE in the presence of 2-mercaptoethanol, showed the
presence of two polypeptides with $M_c = 185,000$ and 179,000 (Fig. 5). The presence of 8 M urea during electrophoresis provides better resolution of these two chains (Fig. 5B). A spectrophotometric scan revealed a weight ratio of 2:1 between the 185,000 and 179,000 polypeptide bands (Fig. 6, lower profile).

Bacterial collagenase digestion of thiol-solubilized Ascari
collagen resulted in a resistant fragment(s) which exhibited a
single band on SDS-PAGE, after reduction, corresponding to
$M_r = 30,000$ (Fig. 7). Rotary-shadowsing electron microscopy
allowed the identification of two collagenase-resistant parti-
cles which were globular in shape. The mean diameter of the
major species was $17 \pm 3$ nm (35 molecules) and that of the
minor species was $23 \pm 2$ nm (35 molecules). The mean
diameter of each species was the same regardless of whether
it was sprayed at pH 3.5 or 7.5.

The chemical composition of thiol-solubilized Ascari
collagen is presented in Table II. Its collagenous character is
shown by the high content of glycine, hydroxylysine, hydroxy-
proline, and proline. The glycine content, however, is less
than 33 mol % and this, along with the presence of half-
cystine residues, suggests a procollagen-like structure. Less
than 50% of the half-cystine residues are carboxymethylated,
indicating that less than half of the disulfide bonds in mole-
cules are reduced under the conditions employed for solubili-
ization. The carbohydrate residues appear to be located in the
disaccharide unit, glucosylgalactosylhydroxylysine, and in a
heteropolysaccharide unit, both of which are present in A.
suum intestinal basement membrane (17).

Characterization of Pepsin-solubilized Collagen and Com-
parison to Thiol-solubilized Collagen—Ascari
collagen was
excised from the intact membrane by pepsin hydrolysis and
then examined by electron microscopy and SDS-PAGE. The

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**Fig. 1.** Scheme for the solubilization of $\alpha$-1 and $\alpha$-2 chains of A. suum intestinal basement membrane under conditions which preserve the triple-helical conformation. Yields based on dry weight measurements are presented in parentheses at each step. Inset, SDS-PAGE analysis of solubilized $\alpha$-1 and $\alpha$-2 chains after reduction of disulfide bonds in SDS. A, whole Ascaris membrane; B, solubilized $\alpha$-1 and $\alpha$-2 chains.

**Fig. 2.** Gel filtration of thiol-solubilized Ascaris collagen. Twenty mg of protein in 10 ml of 0.1 M Tris-HCl, 0.5 M NaCl, pH 8.3, was applied to a 2 x 90-cm column of Sephacryl S-300 equilibrated in the buffer at 4 °C and eluted at a flow rate of 12 ml/h. Fractions of 2.5 ml were collected and their relative protein concentrations determined by absorbance measurements at 280 nm. Fractions denoted by the bar were found to be homogeneous in $\alpha$-1 and $\alpha$-2 chains by SDS-PAGE in the presence of a reducing agent (Fig. 5).
**Fig. 3.** Electron micrographs of various *Ascaris* collagen samples. Panels A and B represent thiol-solubilized *Ascaris* monomers in the pooled fractions from Sephacryl S-300 (Fig. 2). Panels C and D show thiol-solubilized dimers from the same pooled fractions. Panel E is a micrograph of a typical pepsin-solubilized dimer; note the loop in the middle of the molecule which indicates a highly flexible region. Panel F illustrates thiol-solubilized collagen monomers and dimers after pepsin treatment; only monomer-sized molecules were obtained. The globular particles in panel F are pepsin molecules, as shown by a control experiment. The total magnification of each micrograph is 240,000. Each bar has a length equivalent to 100 nm.

**Table I**

Dimensions and flexibility of collagenous molecules of *A. suum* basement membrane

Refer to the text for more detail. The contour length and end-to-end distance are given as the average ± the standard deviation for the number of molecules indicated in the bottom row. The standard deviation in the contour length and end-to-end distance for an individual molecule is 1.7% as determined from 25 measurements on a single molecule. Length and distance are in nanometers. Representative electron micrographs are shown in Fig. 3. The schematic drawings in the first row of the table depict the gross conformations of the molecules.

| Schematic drawing | Thiol-solubilized dimer | Pepsin-solubilized dimer | Thiol-solubilized monomer | Pepsin-treated thiol-solubilized collagen |
|-------------------|-------------------------|--------------------------|---------------------------|------------------------------------------|
| Contour length, \(L\) | 918 ± 24               | 835 ± 42                | 469 ± 21                  | 417 ± 53                                 |
| Root-mean-square end-to-end distance, \(\langle R^2 \rangle^{1/2}\) | 486                    | 389                     | 298                       | 371                                      |
| End-to-end distance, \(R\) | 444                    | 368                     | 286                       | 368                                      |
| Persistence length, \(a\) | 155 ± 27               | 103 ± 20                | 129 ± 21                  | 563 ± 133                                |
| \(\langle R^2 \rangle^{1/2}/L\) | 0.53                   | 0.47                    | 0.64                      | 0.89                                     |
| \(|((\langle R^2 \rangle^{1/2}/L)-(R/L))_i| average for the sample; \(i\) refers to individual molecules | 0.18                    | 0.18                     | 0.15                       | 0.08                                      |
| Number of molecules | 25                     | 24                      | 24                        | 23                                       |

Microscopy results are summarized in Table I, and a representative molecule is shown in Fig. 3E. The dimeric species was again found to be the major component (2, 8). It has a contour length of 835 ± 42 nm, which is about 80 nm less than that of the thiol-solubilized dimer, indicating cleavage site(s) on each end of the molecule. The greater part of the triple-helical domain of thiol-solubilized collagen must be identical to the triple-helical domain of pepsin-solubilized collagen because thiol solubilization yields 70% of the total *Ascaris* membrane collagen, and pepsin solubilization yields 90% of the collagen (8).

The molecular weight of the pepsin-solubilized dimer was calculated from its mean contour length of 835 nm, the mean residue weight of 97 which was calculated from its amino acid composition (Table III), and carbohydrate content of 11%, the value previously found for pepsin-solubilized *Ascaris* collagen (8). The molecular weight obtained was 955,000, which is in good agreement with the value of 1,010,000 determined
TABLE I

Chemical composition of the constituent chains of thiol-solubilized collagen of the intestinal basement of A. suum before and after hydrolysis with pepsin

| Component | Thiol-solubilized collagen | Pepsin-treated thiol-solubilized collagen |
|-----------|-----------------------------|------------------------------------------|
| Amino acid | Residues/1000 amino acid residues | Residues/1000 amino acid residues |
| 4-Hydroxyproline | 101.1 | 116.9 |
| Aspartic acid | 57.8 | 52.9 |
| Threonine | 30.8 | 15.2 |
| Serine | 39.0 | 19.4 |
| Glutamic acid | 90.1 | 84.5 |
| Proline | 76.2 | 94.4 |
| Glycine | 283.5 | 297.5 |
| Alanine | 57.8 | 61.0 |
| Half-cystine | 5.8 | 2.2 |
| CM-cysteine | 1.4 | 5.8 |
| Valine | 38.1 | 20.6 |
| Methionine | 12.0 | 12.7 |
| Isoleucine | 27.5 | 22.5 |
| Leucine | 63.1 | 75.1 |
| Tyrosine | 13.5 | 10.1 |
| Phenylalanine | 16.4 | 16.9 |
| Hydroxylysine | 25.9 | 31.1 |
| Lysine | 19.8 | 17.0 |
| Histidine | 11.1 | 6.1 |
| Arginine | 42.9 | 38.8 |
| Total | 1000.0 | 1000.0 |
| Carbohydrate | | |
| Fucose | 5.5 | |
| Mannose | 6.3 | |
| Glucose | 26.7 | |
| Galactose | 39.3 | |
| Glucosamine | 8.8 | |

- Determined as the sum of half-cystine and cysteic acid.
- Carboxymethylcysteine.
- Sum of methionine, methionine sulfoxide, and methionine sulfoxide.

Fig. 4. SDS-agarose gel electrophoretic analysis of thiol-solubilized and pepsin-solubilized Ascaris collagen. Gel A, type I calf skin collagen; the molecular mass of the α chain is 95 kDa, that of β is 190 kDa, etc. Gel B, thiol-solubilized Ascaris collagen monomers (615 kDa) and dimers (1080 kDa) in pooled fractions from Sephacryl S-300 (Fig. 2); Gel C, thiol-solubilized monomers and dimers after pepsin treatment (435 kDa). Gel D, pepsin-solubilized dimer (910 kDa), which represents 90% of Ascaris intestinal basement collagen (2, 8).

Fig. 5. SDS-PAGE analysis of the constituent chains of thiol-solubilized and pepsin-solubilized Ascaris collagen after reduction of disulfide bonds. Panel A, 5% polyacrylamide gels in the absence of urea. Gel 1 represents whole intestinal basement membrane; gel 2, α-1 and α-2 chains of the thiol-solubilized collagen of pooled fractions from Sephacryl S-300 (Fig. 2); gel 3, peptic digest of whole Ascaris intestinal basement membrane; gel 4, pepsin-treated thiol-solubilized collagen; gel 5, type I collagen from calf skin. Panel B is the same as panel A except that 8 M urea was incorporated into the gel and sample buffer to improve resolution.

Fig. 6. Spectrophotometric scans of gels displayed in Fig. 5B. The lower profile is the scan of gel 2 (Fig. 5B) which represents the constituent chains of thiol-solubilized collagen. The upper profile is the scan of gel 4 (Fig. 5B) which represents the constituent chains of pepsin-treated thiol-solubilized collagen.

previously by sedimentation equilibrium in a nondenaturing solvent (2).

SDS-PAGE analysis of pepsin-solubilized collagen after reduction of disulfide bonds showed that the constituent chains are smaller than those of thiol-solubilized collagen (Fig. 5, gel 3 compared with gel 2). Two major chains were found, as before (2, 8), which have apparent molecular weights of 158,000 and 153,000, as compared with 185,000 and 179,000 for thiol-solubilized collagen.

The pepsin-solubilized dimer lacks the globular domain that is seen in the central region of the thiol-solubilized dimer (Fig. 3, C and D). However, this does not indicate whether the linkage between monomers in the pepsin-solubilized dimer...
occurs between (previously) globular ends or between the nonglobular ends. To further study the relationship between thiol-solubilized and pepsin-solubilized *Ascaris* collagen, we characterized the changes in the former caused by exposure to pepsin. We wished to see if this would produce fragments identical to those obtained by the direct action of pepsin on the membrane. Examination of pepsin-treated thiol-solubilized collagen by electron microscopy (Table I and Fig. 3F) revealed loss of the globular domains and conversion of both the dimers and monomers into threadlike particles which are shorter than thiol-solubilized monomer. The mean contour length of the thiol-solubilized collagen was 417 ± 53 nm after pepsin treatment as opposed to 469 ± 21 nm for the thiol-solubilized monomer before pepsin digestion. The value 417 nm is almost exactly half that of the length of pepsin-solubilized dimer. Furthermore, the calculated molecular weight of pepsin-treated thiol-solubilized *Ascaris* collagen is 485,000, which is quite close to the value of 520,000 found previously by sedimentation equilibrium measurements when the pepsin-solubilized dimer was reduced under non-denaturing conditions (2). These results show that solubilization of *Ascaris* collagen with 2-mercaptoethanol followed by pepsin treatment yields a product which is almost identical to that obtained by reversing the order in which the reagents were used. These results are a further indication that the thiol-solubilized and pepsin-solubilized dimers were derived from the same supramolecular assembly of collagen molecules.

SDS-agarose gel electrophoresis also showed that pepsin digestion of thiol-solubilized collagen generated one major product (*M* = 435,000; Fig. 4, gel C). The amino acid composition indicates that it is relatively more collagenous than the parent molecule. However, residual noncollagenous sequences must still be present since glycine constitutes less than one-third of the amino acid residues (Table II). Furthermore, the amino acid composition is identical to that of pepsin-resistant segments generated by digestion of whole *Ascaris* membrane, which were characterized in a previous study (8). After denaturation and reduction, the products migrated in SDS-polyacrylamide gels as two major polypeptides (*M* = 158,000 and 153,000) and one minor (*M* = 140,000) as seen in Fig. 5, gel 4 in panels A and B. On the basis of relative concentrations obtained from scans of SDS-polyacrylamide gels (Fig. 6, upper profile), it appears that the α-1 chain generates the *M* = 158,000 product and the α-2 chain generates the *M* = 153,000 product; these are designated α-1 (pepsin) chain and α-2 (pepsin) chain, respectively. The minor product (*M* = 140,000) presumably represents further cleavage of the α-1 (pepsin) chain or the α-2 (pepsin) chain, or both. In addition, each of the chains has an electrophoretic mobility which is identical to that of the corresponding chain of pepsin-solubilized *Ascaris* collagen (Fig. 5, gel 3 compared to gel 4).

These results indicate that the products obtained by first solubilizing *Ascaris* collagen with 2-mercaptoethanol and then treating it with pepsin are quite similar to those produced by solubilization with pepsin followed by reduction of disulfide bonds. It should be noted, however, that the order in which the protease and reductant are used does cause some differences in the product obtained. For example, extraction of the membrane with the thiol yields a mixture of monomers and dimers. These are converted into a shortened monomeric triple helix upon digestion with pepsin. On the other hand, direct treatment of the membrane with pepsin yields primarily a dimeric species. There are two alternative explanations for these results. The dimer may be produced by complete excision of the globular domains; the intermolecular disulfide bonds, then, would involve the nonglobular ends. On the other hand, the intermolecular disulfide bonds might involve the globular ends, in which case they would be part of a residual structure left after pepsin hydrolysis of native collagen molecules in the basement membrane. To explain, then, the cleavage of the thiol-solubilized dimer into monomers by pepsin, it is necessary to invoke the creation of new pepsin-sensitive sites in the globular domains by reduction of some of the disulfide bonds in these regions. Indeed, such sites have been reported in type IV collagen (18, 19).

Pepsin digestion *per se* may result in fragments that are somewhat heterogeneous simply because of incomplete specificity with respect to the peptide bonds that it cleaves. Inspection of Table I shows that the pepsin-treated *Ascaris* collagens (columns 2 and 4) have greater percentage standard deviations in their contour lengths than the samples (columns 1 and 3) not treated with pepsin. The distribution of molecular size is not seen by SDS-PAGE or SDS-agarose electrophoresis because their resolving power is not as great as that of electron microscopy which enables one to examine individual molecules.

**Flexibility of Collagen Molecules**—The electron micrographs of *Ascaris* basement membrane collagen indicate that it is similar to other collagens in that it is a somewhat flexible polymer, rather than a rigid rod. The flexibility is apparent in Fig. 3, where we present two extreme gross conformations of the thiol-solubilized monomer (panels A and B) and dimer (panels C and D). To quantitate flexibility, we measured the distribution of the absolute value of the end-to-end distance, *R*, and the contour length, *L*, for each of the forms of *Ascaris* collagen described above. As a simple empirical measure of flexibility, we calculated the ratio of the root-mean-square end-to-end distance to the average contour length for a given sample. The value of (*R*²)¹/²/*L* is 1.0 for a monodisperse sample of straight rigid rods; lower values indicate flexibility, nonlinearity, or both. In addition, we calculated the average of the absolute value of the difference between (*R*²)¹/²/*L* and *R*/*L* for

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**Fig. 7. SDS-PAGE analysis of products from collagenase digestion of thiol-solubilized collagen (pooled fractions, Fig. 2).** Samples were reduced in SDS and electrophoresed using system IV (see “Experimental Procedures”). Gel A, thiol-solubilized collagen incubated without collagenase; gel B, after collagenase digestion. Gel C, *M*ₙ standards (1, bovine serum albumin; 2, pepsinogen; 3, trypsinogen; 4, β-lactoglobulin; 5, lysozyme). The electron micrograph shows the major collagenase-resistant domain, which has a diameter of 17 nm. The total magnification is 240,000. The bar has a length equivalent to 190 nm.
individual molecules, \((R/L)\), i.e. \(|<(R^2)/L> - (R/L)\)| averaged over all molecules of the sample. The value of this quantity is zero for a straight rigid rod and other values indicate flexibility; the value increases with the degree of flexibility. The results of this analysis are presented in Table 1. The pepsin-treated thiol-solubilized collagen is straighter and less flexible than the other forms, as it has a value of \(<(R^2)/L>\) of 0.89 and an average of \(|<(R^2)/L> - (R/L)\)| of 0.68, whereas the corresponding pairs of values for the thiol-solubilized monomer and dimer and the pepsin-solubilized dimer are 0.64, 0.15; 0.53, 0.18; and 0.47, 0.18, respectively. Now considering the pepsin-solubilized dimer, it contains two rigid subunits but has a high overall degree of flexibility. This can be attributed to flexibility in the center of the dimer. As is seen in the representative electron micrograph (Fig. 3E), the majority of the pepsin-solubilized dimers contain a loop in the center of the molecule. Thus, the flexibility is chiefly due to a “swivel” that permits rotation of the subunits about each other.

The pepsin-solubilized dimer is more flexible than the thiol-solubilized dimer. This is due to a balance between two factors. On one hand, the thiol-solubilized dimer contains a bulky globular domain in its center, which the pepsin-solubilized dimer lacks. On the other hand, pepsin has removed flexible regions from the largely triple-helical threadlike portions of thiol-solubilized collagen, as is revealed by the large increase in \(<(R^2)/L>\) and decrease in \(|<(R^2)/L> - (R/L)\)| after pepsin treatment.

To compare *Ascaris* collagen to other collagens by electron microscopy, we calculated the persistence length by use of the following equation (20),

\[
\langle R^2 \rangle = 2a^2 (L/a - 1 + e^{-a})
\]

where \(\langle R^2 \rangle\) is the mean square end-to-end distance, \(L\) is the average contour length, and \(a\) is a stiffness parameter called the persistence length; \(a\) may have values much less than \(L\) for very flexible chains and values much greater than \(L\), even approaching infinity, for straight rigid rods. The equation was derived for linear polymers made of segments which can rotate about their neighbors in a three-dimensional mode. The equation was thus designed for flexible polymers in solution, but if the collagen molecule is rigid under conditions which preserve the triple-helical conformation, electron microscopy should result in values of the persistence length that are rough approximations to those that would be obtained by use of solution-based methods (21, 22). We found values of \(a\) of 155 ± 27 and 129 ± 21 nm for thiol-solubilized dimeric and monomeric *Ascaris* collagen, respectively. These values are within experimental error of the value of 114 ± 15 nm found by Fessler et al. (11) for a 270-nm type IV collagen fragment. The values that we obtained are quite close to the value of 114 ± 10 nm for type I collagen and are similar to the value of 80 ± 8 nm for type III collagen found by Hoffman et al. (22); their published values were multiplied by 2 because their calculations were made with use of an equation for a two-dimensional model that yields persistence lengths half as large as those obtained from the equation for the three-dimensional model. Types I and III collagen have been found by solution-based methods to be flexible (see, for example, Ref. 23), and our comparison of persistence lengths confirms that this is also true for *Ascaris* basement membrane collagen.

In a previous report (2) on the characterization of pepsin-solubilized *Ascaris* collagen, we proposed a model in which two triple-helical units of equal length were joined end-to-end by disulfide bonds. We recognized that our model, which was based in large part on interpretation of the value of the translational frictional coefficient, could not take flexibility into account. For example, flexibility such as that due to free rotation of the axis of one cylindrical subunit about the axis of the other would have only a minor effect on the frictional coefficient. Silver and Birk (23) have recently shown that other modes of bending also have little effect on the translational frictional coefficients of threadlike molecules. The flexibility of *Ascaris* collagen was clearly shown in the present study by electron microscopy and is an important addition to the previous model. Although the functional role of flexibility is uncertain, it is conceivable that it permits adjustment of the geometry of a triple-helical monomer to the geometry of other monomers, other kinds of proteins, and proteoglycans, with which it interacts to form the basement membrane. The flexibility of collagen may, in part, cause the completed basement membrane to be flexible and allow it to adjust its shape during the functioning of the intestine of *Ascaris*.

**DISCUSSION**

In the work reported here, we developed a chemical extraction procedure which enabled us to solubilize 70% of the collagenous components of the intestinal basement membrane of the helminth *A. suum* under conditions which preserve the triple-helical conformation. This procedure utilizes selective reduction of disulfide bonds with a thiol. An advantage of this method over pepsin solubilization is that its use results in collagenous proteins which are longer and somewhat more homogeneous than those obtained by pepsin hydrolysis (2, 8). Also, this method allows preservation of a noncollagenous globular region, located at one of the ends of a collagen molecule, which would be destroyed by pepsin. In this study we found that two of these domains are juxtaposed in the middle of a dimer made of two triple-helical subunits which are joined by disulfide bonds. The thiol-solubilized collagen was characterized with respect to its size, shape, molecular organization, and chemical nature of its constituent chains as summarized in Fig. 8.

Thiol-solubilized collagen contains two kinds of polypeptide chains which we have designated \(\alpha-1\) and \(\alpha-2\) chains; they were identified as collagogenous constituents in a previous study (17). These polypeptides share several characteristics with pro-\(\alpha-1\) (IV) and pro-\(\alpha-2\) (IV) chains from vertebrate tissues (3, 24–27, 29, 30). Amino acid analysis (Table II) revealed high levels of hydroxyproline and hydroxylysine, low levels of alanine and arginine, the presence of half-cystine, and a glycine content of less than 35 mol%. This composition suggested a procollagen-like structure which contains noncollagenous segments. We demonstrated the latter by finding a collagenase-resistant segment(s) of \(M_c = 30,000\). This fragment is close in molecular weight to carboxyl-terminal segments observed after collagenase digestion of type IV procollagen. Additional similarity was revealed by electron microscopy which showed that the segments form a globular aggregate, as do the type IV fragments (3, 10, 26, 30).

*Ascaris* collagen chains have molecular weights of 185,000 and 179,000 and thus correspond in size to pro-\(\alpha-1\) (IV) and pro-\(\alpha-2\) (IV) chains (24, 25, 28). Upon pepsin digestion the two chains yield collagenous polypeptides of \(M_c = 158,000\) (\(\alpha-1\) (pepsin)) and 163,000 (\(\alpha-2\) (pepsin)) which appear to be similar to pepsin-resistant segments generated from type IV procollagen (3, 22, 26, 31). These are identical in size to the collagenous segments (a and b) generated by pepsin digestion of whole *Ascaris* membrane as previously reported (8). They are more collagenous in nature than the \(\alpha-1\) and \(\alpha-2\) chains, indicating the removal of noncollagenous segments by pepsin. Our electron microscopy results show that one of these seg-
ments is the collagenase-resistant globular domain and one is located at the other end of the collagen molecule.

A supramolecular organization of type IV collagen has been proposed in which four type IV collagen molecules interact through their amino-terminal domains to form a tetrameric structure. The interaction of the globular domains of two type IV collagen molecules then results in the establishment of a lattice network (5, 6, 10, 11, 32). These associations involve noncovalent interactions, disulfide bonds, and lysine-derived cross-links. There is no a priori reason to assume this model would encompass all modes of collagen association in mammalian basement membranes or for it to apply without modification to invertebrate systems. It is of interest, with this caveat in mind, to relate our results for Ascaris basement membrane collagen to the lattice model for mammalian systems. We have found that the major collagenous structural unit of Ascaris membrane is strikingly like that of mammalian membranes in that it is a dimer made of thread-like monomers of equal length joined at globular ends through disulfide bonds. We do not have direct evidence that these globular domains are located at the carboxyl-terminal ends as is the case with mammalian collagen. They both, however, have the same diameter and are made of M, 30,000 segments of the constituent chains; this is suggestive of homology. Neither do we have direct evidence for the existence of linking structures involving the amino-terminal regions. Inspection of collagenase and pepsin digests of Ascaris intestinal basement membrane by electron microscopy did not reveal any fragments which correspond in size or shape to any of the reported tetrameric amino-terminal cross-linking structures, commonly designated as S regions, prepared by the use of these enzymes (3, 6, 10, 11), and they would have been easy to detect. The presence of such structures is suggested, though, by the ability of either 2-mercaptoethanol, which reduces disulfide bonds, or the protease pepsin to solubilize the collagenous domain. Perhaps the Ascaris cross-linking moiety is susceptible to cleavage by collagenase and pepsin, in contrast to its mammalian counterpart. It can be shown that the presence of a single pepsin-sensitive region in the amino-terminal cross-linking portion of each collagen molecule can give rise to dimeric structures instead of tetrameric fragments. Other supramolecular structures could give rise to dimer molecules identical with or similar to that which we characterized. For example, Ascaris collagen could exist in the native membrane as a linear polymer in which the monomeric triple helices are associated in a head-to-head (globule-to-globule) and tail-to-tail fashion, i.e., etc. These could, theoretically, form matrices by means of noncovalent interactions. The thiol-solubilized dimer could be extracted then only if disulfide bonds in the tail regions were broken by the action of the tiol. On the other hand, a pepsin-solubilized dimer could be generated in either of two ways: 1) by cleavage of peptide bonds in all three chains in their tail regions and substantial but incomplete hydrolysis of the head regions, leaving cross-links between triple-helical subunits; or 2) by cleavage of all three chains in the head regions, with cross-links being retained in the tail regions after hydrolysis.

Fessler et al. (12) have found that Drosophila basement membrane collagen obtained from cell cultures consists largely of 465-nm triple-helical threadlike particles which have a globular knob at one end. This structure is quite similar to that of Ascaris basement membrane collagen. Drosophila collagen can self-associate, primarily into dimers, and to a lesser extent into trimers and tetramers, through disulfide bond formation between nonglobular ends. Their results along with ours and those on mammalian systems show that association sites at each end of the basement membrane

**Fig. 8. Schematic summary of the isolation and characterization of A. suum intestinal basement membrane with respect to chemical and physical properties and molecular organization.** Two collagenous molecules were solubilized under conditions previously demonstrated to preserve the triple-helical conformation. These were shown to exist in a monomer-dimer relationship. The constituent chains (α-1 and α-2) of each triple helix contain both collagenous (denoted as →) and noncollagenous (←) segments. Peptic digestion (right side of figure) converts these polypeptides into forms which are more collagenous (designated α-1 (pepsin) and α-2 (pepsin)). This involves removal of a noncollagenous segment(s) which corresponds in size to the collagenase-resistant product(s), shown in the left side of the figure. Production of a monomer by pepsin digestion of the thiol-solubilized dimer might require that some of the disulfide bonds (not indicated in the figure) be reduced in order to make certain bonds sensitive to pepsin. This would allow excision of regions that contain intermolecular disulfide bonds.
collagen molecule existed in more primitive organisms and were conserved during evolution.

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