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Orientation of Type VI Collagen Monomers in Molecular Aggregates†

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ABSTRACT: Type VI collagen, prepared from guanidine extracts of human amnion, contains very little monomeric material, the major forms being dimers and tetramers. In order to study the orientation of the molecules in these aggregates, they were digested with pepsin followed by bacterial collagenase. Two fragments were isolated, one containing part of the inner globular domain still attached to part of the triple helix and the other containing large fragments of the outer globular domain. Each fraction was further analyzed; peptides were isolated and their amino-terminal amino acid sequences determined. By comparing the determined sequences with published data, it was found that the outer globular domain contained sequences derived from the amino-terminal domain of all three chains of type VI collagen whereas the inner globular domain contained sequences from the carboxy-terminal domain. This provided direct chemical evidence that dimers and tetramers of type VI collagen are formed by overlapping carboxy-terminal regions of the monomers.

Type VI collagen chains were first isolated from the pepsin-solubilized intimal layer of human blood vessel (Chung et al., 1976). Since then, native pepsin-solubilized type VI collagen has been isolated and characterized from a variety of tissues including placenta, cornea, and ligamentum nuchae [for a review, see Timpl and Engel (1987)]. A structural model for type VI collagen filaments was proposed by Furthmayr et al. (1983) based upon electron microscope studies of rotary-shadowed native and the partially reduced pepsin-solubilized type VI collagen. Type VI collagen monomers contain three different polypeptide chains (Jander et al., 1981, 1983) which form a central 105-nm-long triple-helical region with globular domains at each end. Both intrachain and interchain disulfide bonds are present within the

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monomers (Rauterberg et al., 1982; Odermatt et al., 1983). The major portion of material solubilized from tissue is in the form of dimers or tetramers. Type VI collagen dimers are formed by lateral aggregation of two antiparallel monomers with their helical rods overlapped by about 75 nm (Figure 1). Tetramers, the basic unit of type VI collagen filaments, are formed by the aggregation of two dimers in a parallel fashion, with their ends in register. Intermolecular disulfide bonds and perhaps other covalent cross-links are responsible for further stabilizing the dimers, tetramers, and filaments of type VI collagen (Odermatt et al., 1983; Engel et al., 1985). Evidence for an antiparallel alignment of molecules in dimers and tetramers was provided by studying complexes of monoclonal antibody with pepsin-solubilized type VI collagen. The epitope of the monoclonal antibody was located on the triple-helical region, approximately 20 nm away from the larger globular domain which corresponds to the inner globular domain of dimers and tetramers (Linsenmayer et al., 1986). All of the antibody–dimer/tetramer complexes observed had epitopes located in the overlapping helical domains, indicating an antiparallel arrangement of monomers.

The intact tissue form of type VI collagen has been extracted from several tissues using guanidine (Jander et al., 1984; Gibson & Cleary, 1985; Ayad et al., 1985). The apparent molecular weights of intact α chains vary somewhat depending upon species (Trüeb & Winterhalter, 1986). Human type VI collagen contains two chains with a molecular weight of about 140K and a third of M_r 260K (Engvall et al., 1986). Unlike pepsin-solubilized type VI collagen, intact molecules have large globular domains at each end of the helix which are of approximately equal size (Jander et al., 1984). Pepsin preferentially digests the outer globular domain of type VI collagen dimers and tetramers. As only a few nontriplet (Gly-X-Y) amino acids sequences were found in the N-terminal sequences of pepsin-solubilized type VI collagen α chains (Chu et al., 1987), it was suggested that the outer globular domains of type VI collagen dimers and tetramers may be the N-terminus of type VI monomers. However, this was not conclusive evidence as the inner globular domain may be damaged by pepsin digestion but held together by disulfide bonds, giving only the appearance that an extended noncollagenous sequence is still attached to the helix.

The assembly of type VI monomers into dimers and tetramers occurs intracellularly (Engvall et al., 1986) and is possibly guided by the affinity of one of the globular domains for a binding site on an adjacent triple helix. Tetramers associate extracellularly to form linear filaments, probably guided by specific interactions between the outer globular domains. In order to investigate these possible interactions, the orientation of the type VI collagen monomer in its dimeric and tetrameric units must first be determined. A method was therefore developed to directly isolate the inner and the outer globular domains of pepsin-treated type VI collagen, and peptides purified from both globular domains were sequenced (Figure 1). Because native intact type VI collagen is resistant to digestion with bacterial collagenase, a direct approach as used for other collagens was not possible, and a new approach had to be developed. The amino acid sequence data presented here provide direct chemical evidence that the carboxy end of monomers overlaps to form higher aggregates.

Experimental Procedures

Materials

Human placental tissues were obtained from the Oregon Health Sciences University. Benzamidine, ϵ-amino-*n*-caproic acid, *N*-ethylmaleimide, phenylmethanesulfonyl fluoride, EDTA, and urea were purchased from Sigma. Urea was deionized by using the mixed-bed ion exchanger M-614 from Baker. Ultrapure guanidine hydrochloride (Gdn-HCl) was from Amresco and Sephacryl S-500 (superfine) from Pharmacia Biotechnology, Inc. Bacterial collagenase (CLSPA) was from Cooper Biochemical Inc. and repurified on a Sephacryl S-300 (superfine, 0.8 cm × 110 cm) column. The C_4 (10 mm × 250 mm; Vydac 214TP510) and C_{18} (4.6 mm × 250 mm; Vydac 218TP54) reverse-phase columns for high-performance liquid chromatography were obtained from the Separations Group. Molecular sieve columns, G2000 (600 mm × 7.5 mm) and G3000 (600 mm × 7.5 mm), were purchased from Phenomenex. Bio-Sil TSK-400 (600 mm × 21.5 mm) was from Bio-Rad Laboratories.

Methods

Preparation of Type VI Collagen. (A) Gdn-HCl Extraction. All procedures were performed at 4 °C, and all solutions used contained 10 mM EDTA, 2 mM *N*-ethylmaleimide, 1 mM phenylmethanesulfonyl fluoride, 5 mM benzamidine, and 0.1 M ϵ-amino-*n*-caproic acid. Fresh amniotic membranes of human placenta were washed immediately and stored in 50 mM Tris-HCl buffer, pH 7.0, containing 1 M NaCl at −20 °C. About 200 g wet weight of membranes was thawed overnight at 4 °C and then homogenized in the Tris-HCl buffer described above. The tissue residue was collected by centrifugation and washed with the same buffer once more, followed by two washes with 0.5 M acetic acid. The insoluble residue was extracted overnight with 3 volumes (v/w) of 0.1 M Tris-HCl buffer, pH 7.8, containing 5.5 M Gdn-HCl (final concentration) and centrifuged at 18000*g* for 60 min. The supernatant was dialyzed extensively against 1% acetic acid. The precipitate formed during dialysis was collected by centrifugation and redissolved in 40 volumes (v/w) of the buffer described above but containing 6 M Gdn-HCl. The solution was clarified by centrifugation, and the supernatant solution was again dialyzed extensively against 1% acetic acid. The precipitate formed during dialysis was collected by centrifugation and dissolved in 40 volumes (v/w) of 0.05 M Tris-HCl containing 6 M urea and 0.12 M NaCl, pH 8.3 (DEAE buffer), dialyzed against 2 L of the same buffer, and clarified by centrifugation. DEAE-cellulose (one-third of the sample volume), which had been equilibrated with the same buffer, was stirred into the supernatant solution. Proteins which did not bind to the cellulose were collected, concentrated with Bio-Gel concentrator resin, and chromatographed on a Sephacryl S-500 column (80 cm × 5 cm) equilibrated at room temperature with 0.04 M Tris-HCl buffer, pH 6.8, containing 6 M urea and 0.1 M Na_2SO_4 (TSK buffer). Column fractions which contained type VI collagen, identified by monoclonal antibody, gel electrophoresis, and their electron micrographs after rotary shadowing, were pooled, dialyzed against 1% acetic acid, lyophilized, and stored at −80 °C. The final yield of type VI collagen was approximately 0.5% of acid-extracted tissue residue (w/w). Depending upon the requirements of experiments, type VI collagen was further purified by using two Bio-Sil TSK-400 columns (60 cm × 2.15 cm) connected in tandem and equilibrated with TSK buffer.

(B) Pepsin-Digested Type VI Collagen. The lyophilized purified Gdn-HCl-extracted type VI collagen was dissolved in the TSK buffer to a final concentration of 1 mg/mL and dialyzed extensively against 0.5 M acetic acid. Pepsin (0.01 of substrate) was added to the dialysate, and the solution was incubated at 4 °C for 24 h. The reaction mixture was frozen, lyophilized, redissolved in TSK buffer, and chromatographed

on a Sephacryl S-500 column as described under section A.

(*C*) *Preparation of Bacterial Collagenase Digested Native Type VI Collagen Fragment* (*CF*). Lyophilized material of purified pepsin-digested type VI collagen was dissolved into TSK buffer to give a final concentration of 1 mg/mL and dialyzed extensively against 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $CaCl_2$, 0.4 M NaCl, 5 mM benzamidine, 2 mM *N*-ethylmaleimide, and 1 mM phenylmethanesulfonyl fluoride. The digestion was performed overnight at 37 °C with addition of 5 μL of a purified bacterial collagenase solution per 100 μL of sample. The reaction was terminated by adding 10 mM EDTA and the digest dialyzed extensively against 1% acetic acid and lyophilized. The peptide mixture (40 mg) was dissolved in 10 mL of TSK buffer and fractionated over two Bio-Sil TSK-400 columns (60 cm × 2.15 cm) connected in tandem and equilibrated with TSK buffer.

Peptide Purification. Peptides generated by bacterial collagenase from either of the globular domains were purified on a reverse-phase C_4 (Vydac 214TP510) or a C_{18} (Vydac 218TP54) column depending on experiments. The column was equilibrated with 0.1% trifluoroacetic acid (A solution) and eluted with 0.08% trifluoroacetic acid containing 60% 2-propanol (B solution). The column was thermostated at 50 °C. The flow rate was 1.5 mL/min for the C_4 column and 1 mL/min for the C_{18} column.

Analytical Methods. Reduction of disulfide bonds, using β-mercaptoethanol, and blocking of thiol groups with vinylpyridine were performed as described by Friedman et al. (1970). Prior to the reduction, protein was dissolved in 0.5 M Tris-HCl buffer, pH 7.5, containing 8 M urea, 0.2 M NaCl, and 5 mM EDTA.

Amino acid analyses were carried out using the Pico Tag system (Waters) with slight modification (Morris et al., 1986).

Peptide sequences were determined by using a gas-phase sequencer (Applied Biosystems, Model 470A) with an online PTH-amino acid analyzer (Applied Biosystems, Model 120). The procedures used were those described in the manufacturer manual.

Rotary shadowing of type VI collagen molecules was performed as described earlier (Morris et al., 1986).

SDS gel electrophoresis was performed by using the buffer system described by Laemmli (1970) and a mini slab gradient gel apparatus (Bio-Rad). Molecular weight standards used were myosin heavy chain (200K), galactosidase (116K), phosphorylase (96K), glutamate dehydrogenase (55K), ovalbumin (43K), and lactate dehydrogenase (36K).

Results

The experimental design used is illustrated in Figure 1. The aim was to identify the sequences of the inner and outer globular domains of type VI dimers and tetramers, immediately adjacent to the triple-helical domain. Because dimers and tetramers of type VI collagen are resistant to bacterial collagenase digestion, it was necessary to pretreat first with pepsin. This reduced the size of the inner globular domain considerably and left barely visible remnants of the outer domain. Collagenase digestion removed what was left of the outer domains and the nonoverlapping helical regions, leaving a fragment (CF) containing the inner globular domains and overlapping helices.

Preparation of Native Type VI Collagen. Six peak fractions were obtained on Sephacryl S-500 gel filtration chromatography (Figure 2). Peak II contained type VI collagen contaminated with a trace amount of noncollagenous low molecular weight proteins and type VII collagen which could only be detected by Western blot analysis with type VII collagen

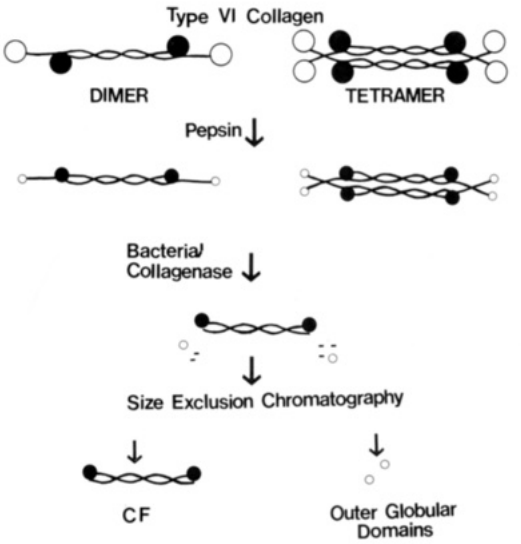

FIGURE 1: This scheme illustrates the steps used to prepare and separate a fragment (CF) from fragments of the outer globular domains of type VI collagen. CF contained part of the inner globular domains and the overlapping region of the helical domain. Note the reduction in size of the globular domains after pepsin treatment and that both dimers and tetramers give rise to the CF fragment. This scheme is based on a model for type VI collagen first proposed by Furthmayr et al. (1983).

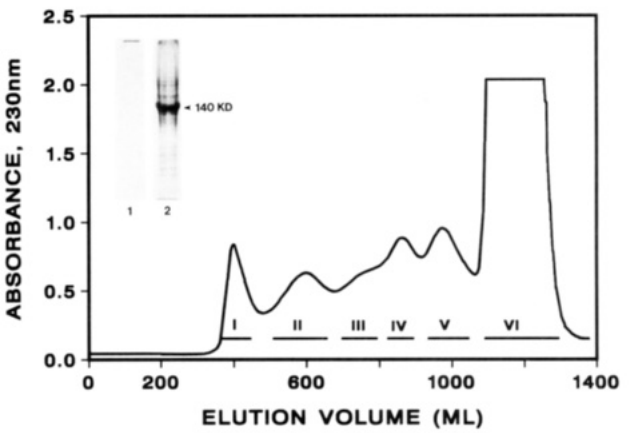

FIGURE 2: Profile of a guanidine extract of human amnion chromatographed on a Sephacryl S-500 (5 cm × 80 cm) molecular sieve column equilibrated with 40 mM Tris-HCl, pH 6.8, buffer containing 100 mM Na_2SO_4 and 6 M urea (TSK buffer) at a flow rate of 90 mL/min. The bars indicated the fractions pooled. Insert: Stained 5–10% SDS–polyacrylamide gel of pool II from the Sephacryl S-500 column; (1) unreduced, (2) reduced sample.

antibodies (Lunstrum et al., 1987). These trace impurities could be removed by further purification on TSK-400 columns (data not shown). Type VI collagen prepared as described is composed of intact α1 and α2 chains which both have apparent molecular weights of 140K (Figure 2, insert). Components with apparent molecular weights around 200K and smaller fragments that migrate just below 140K and around 30–60K were identified in immunoblots with monoclonal antibodies as being derived from the noncollagenous domains of the α3 chain (data not shown). In the unreduced state, all components are disulfide bonded (Figure 2, insert). Electron microscope pictures of rotary-shadowed native type VI show that the bulk of the material is in the form of dimers and tetramers (Figure 4A). The molecules appear to be intact, but as discussed above, there is evidence of proteolytic damage which, for the present investigations, was unimportant.

Preparation of the Collagenase Fragment (*CF*) *of Type VI Collagen.* Native guanidine-extracted type VI collagen (Figure

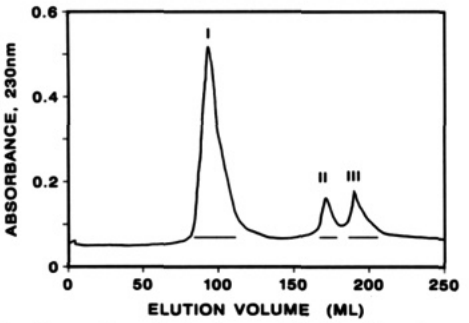

FIGURE 3: Separation of the inner globular domain containing fragment (CF) from the outer globular domains using two preparative TSK-400 columns connected in tandem. Columns (7.5 mm × 600 mm) were equilibrated with TSK buffer (see Figure 2), and the sample was eluted with flow rate at 0.6 mL/min. Fractions I, II, and III were pooled as indicated.

Table I: Amino Acid Compositions of Peptides from the Globular Domains of Type VI Collagen

	residues per 100 for peptides			
amino acid	IG2	IG3	OG2	OG3
Asp	6.6	11.8	6.6	5.0
Glu	7.4	9.6	11.3	7.5
Hyp	2.5	0.4	0	0
Ser	1.3	6.9	7.2	12.0
Gly	9.8	7.4	9.5	15.2
His	0.6	1.0	4.3	2.2
Arg	3.6	7.6	2.5	7.4
Thr	5.4	6.8	3.9	3.0
Ala	3.9	7.6	0	5.3
Pro	3.3	5.3	12.4	5.3
Tyr	ND	0.4	ND	ND
Val	6.3	7.0	11.4	6.8
Met	1.9	0.6	0.4	0
Cys	40.1	2.6	8.6	3.3
Ile	1.0	4.6	6.9	7.2
Leu	1.1	9.8	5.0	3.8
Hyl	0	0	0	0
Phe	5.0	5.4	1.1	2.9
Lys	0.5	5.0	8.7	2.9

4A) was extremely resistant to bacterial collagenase digestion. However, pepsin-digested type VI collagen became partially susceptible to bacterial collagenase. Pepsin treatment digested almost all mass from outer globular domains and left a large proportion of inner globular domains intact (Figure 4B). Subsequent collagenase digestion removed the nonoverlapping helical segments and released the remnant of outer globular domains of tetramers and dimers (Figure 1). The central helices with attached inner globular domains (CF) and the remnant of outer globular domains could be separated on two preparative TSK-400 columns connected in tandem (Figure 3). Three fractions were collected and analyzed by using the electron microscope after rotary shadowing. Peak I contained CF (Figure 4C), but nothing was visualized in peaks II and III. However, gel electrophoresis showed that peaks II and III contained reducible protein fragments, and their amino acid compositions showed that both peaks contained hydroxyproline and hydroxylysine. These fractions were further separated, and cysteine-containing noncollagenous peptides of the outer globular domain were sought.

Peptide Isolation and Sequence. In order to purify the cysteine-containing noncollagenous peptides from the *inner* globular domains, CF was reduced, alkylated, and treated with bacterial collagenase to remove the triplet sequences. Peptides were purified by C_4 reverse-phase column chromatography (Figure 5A). Fractions were pooled and analyzed for their amino acid compositions. Only two peaks, IG2 and IG3 (inner globular 2 and 3), contained cysteine (Table I) and were therefore sequenced. The sizes of peptides IG2 and IG3 were about 5 and 30 kDa, respectively, based on molecular sieve chromatography. The amino acid sequences obtained are shown in Table II and were identified as originating from the carboxy-terminal globular domains of the $\alpha 2$ and $\alpha 3$ chains of type VI collagen from previously published sequences (Chu

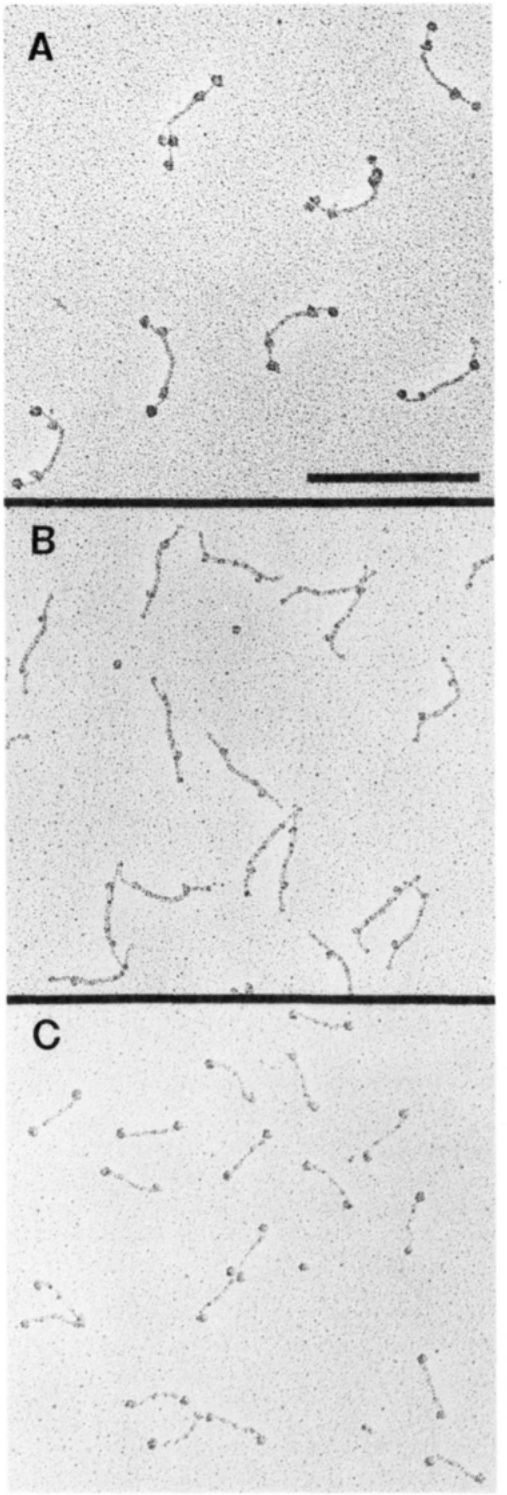

FIGURE 4: Electron micrographs of rotary-shadowed molecules. (A) Purified tissue form of type VI collagen showing the large inner and outer globular domains. (B) Pepsin-treated type VI collagen illustrating the reduced size of the globular domains, particularly the outer domain which is barely visible. (C) Fragment CF from pool I Figure 3 showing the inner globular domains attached to the central overlapping region of the type VI collagen triple helix. Magnification 114000×. Bar = 200 nm.

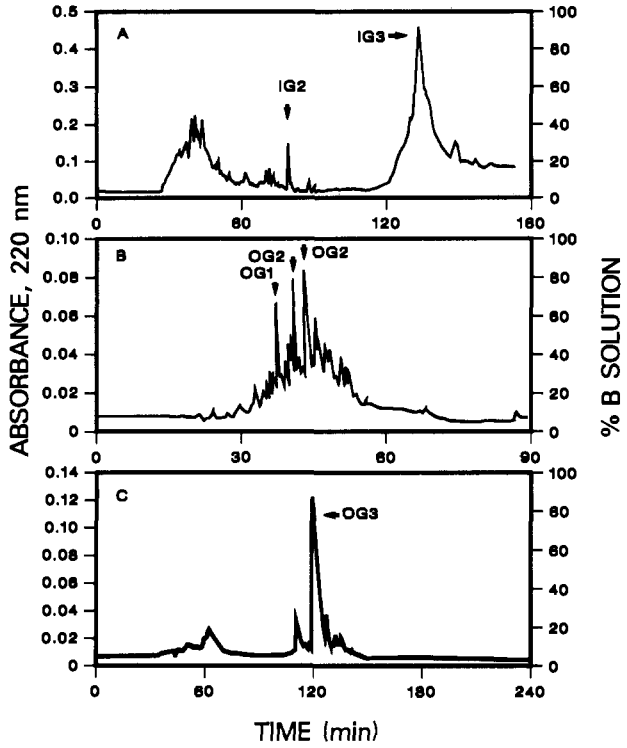

FIGURE 5: Chromatograms illustrating the purification of peptides using reverse-phase high-performance liquid chromatography. Mobile phase: (A) 0.1% trifluoroacetic acid; (B) 0.08% trifluoroacetic acid containing 60% 2-propanol. Temperature, 50 °C. (A) Purification of inner globular domain peptides from a bacterial collagenase digest of CF (peak I of Figure 3) on a Vydac protein C_4 column. Gradient, 0–85% B in 3 h; flow rate, 1.5 mL/min. (B) Peptides purified from the outer globular domains of type VI collagen (peak II of Figure 3) on a Vydac peptide C_{18} column. Gradient, 10–50% B in 90 min; flow rate, 1.0 mL/min. (C) Peptides purified from the outer globular domains of type VI collagen (peak III of Figure 3) on a Vydac peptide C_{18} column. Gradient, 0–60% B in 4 h; flow rate, 1.0 mL/min.

Table II: Amino Acid Sequences of Peptides from the Globular Domains of Type VI Collagen[a]

peptide	amino acids sequence	globular domain	identity
IG2	GPTGDP*GLTECDVMTY-VRETCGCCDCEKRC	inner	α2, C-terminus
IG3	GPRGNRGDSIDQCALIQS-IKDKCPCCYGPL	inner	α3-, C-terminus
OG1	VAFQDCPVDL	outer	α1, N-terminus
OG2	MKHEAYGECYKVSCL	outer	α2, N-terminus
OG3	KACCQGVPC	outer	α3, N-terminus

[a] P* is a symbol used to represent hydroxyproline.

et al., 1988).

The cysteine-containing noncollagenous peptides from the *outer* globular domains were purified from peaks II and III from the molecular sieve column chromatography shown in Figure 3. Peak II was reduced and alkylated before being chromatographed on a C_{18} reverse-phase column (Figure 5B). Three cysteine-containing fractions were sequenced (Table II), two of which (OG2) had identical amino-terminal sequences which originated from the amino-terminal domain of the α2 chain (Chu et al., 1987). The third fraction (OG1) was sequenced and contained a peptide from the amino terminus of the α1 chain (M.-L. Chu, private communication). Peak III was first chromatographed on a C_{18} reverse-phase column (not shown); the cysteine-containing fraction was collected, reduced and alkylated, and rechromatographed on the same column (Figure 5C). One major peak was collected (OG3) and identified by sequence analysis (Table II) as originating from the amino-terminal domain of the α3 chain (Chu et al., 1987).

Discussion

Because of the large noncollagenous domains present in type VI collagen, the molecule is very susceptible to nonspecific proteolysis during preparation. It was found particularly important to store membranes *intact* in the presence of inhibitors and once the membranes were homogenized to proceed with the preparation immediately without interruption. Even with these precautions, the α3 chain was extensively degraded. It is possible that proteolysis takes place in vivo and is part of a physiological process occurring in the amniotic membrane. However, as the sequences required to determine the orientation of the molecules had previously been found at the amino terminus of pepsin-solubilized material (Jander et al., 1983) and the carboxy-terminal sequences used were adjacent to the triple-helical domain, this proteolysis did not interfere with the experiment described here.

The sequences of the carboxy-terminal peptides IG2 and IG3 started with three collagenous triplets which indicated a location carboxy terminal to the triple helix for these sequences. The chains were identified by comparing them with published sequences of type VI collagen (Chu et al., 1988). The amino-terminal sequences of peptides OG2 and OG3 correspond to the amino-terminal sequences of the long form of the α2 and α3 chains of pepsin-solubilized type VI collagen (Chu et al., 1987). The carboxy-terminal peptides of the α1 chain were never found. Possible reasons for this are that the bacterial collagenase peptides were very small due to the prior action of pepsin and were lost during the isolation procedures used or that the noncollagenous ends of this chain were preferentially and totally removed by the action of pepsin. Nevertheless, the ends of the α2 and α3 chains were consistently found in their respective fractions that clearly defined the orientation of the triple helix.

Interstitial collagens, types I, II, III, and presumably V and XI aggregate in a staggered fashion so that the amino-terminal region of one molecule overlaps with the carboxy-terminal region of a neighboring molecule to produce the 4D staggered array typical of the fiber-forming collagens. The helices of basement membrane collagen (type IV) and anchoring fibril collagen (type VII) overlap at their amino-terminal domains. Type VI is the first example of a collagen molecular aggregate being formed by the overlap of the carboxy-terminal regions of the triple helix.

From biosynthetic studies on type VI collagen in fibroblast cultures (Engvall et al., 1986), it appears that dimer and tetramer formation occurs intracellularly. It is possible that the initial associations are guided by the specific interaction of the inner (carboxy) globular domain with a binding site within the triple helix or perhaps specific interactions between the helical domains. Extracellularly, tetramers associate in an end on end fashion to form filaments, probably because of specific interactions between the outer (amino) globular domains. Having determined the orientation of the monomers, these proposed functions for the globular domains can now be further investigated.

Acknowledgments

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Identification and Location of α-Helices in Mammalian Cytochromes P450

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ABSTRACT: A model of the α-helical structure of mammalian cytochromes P450 is proposed. The location and sequence of α-helices in mammalian cytochromes P450 were predicted from their homology with those of cytochrome $P450_{cam}$, and these sequences were generally confirmed as helical in nature by using a secondary structure prediction method. These analyses were applied to 26 sequences in 6 gene families of cytochrome P450. Mammalian cytochromes P450 consist of approximately 100 amino acid residues more than cytochrome $P450_{cam}$. This difference was accounted for by three major areas of insertion: (1) at the N-terminus, (2) between helices C and D and between helices D and E, and (3) between helices J and K. Insertion 1 has been suggested by others as a membrane anchoring sequence, but the apparent insertions at 2 and 3 are novel observations; it is suggested that they may be involved in the binding of cytochrome P450 reductase. Only the mitochondrial cytochrome P450 family appeared to show a major variation from this pattern, as insertion 2 was absent, replaced by an insertion between helices G and H and between helices H and I. This may reflect the difference in electron donor proteins that bind to members of this cytochrome P450 family. Other than these differences the model of mammalian cytochromes P450 proposed maintains the general structure of cytochrome $P450_{cam}$ as determined by its α-helical composition.

Cytochromes P450 form a superfamily of hemoprotein isoenzymes that play a central role in the disposition of a wide variety of endogenous compounds and xenobiotics, many of which are toxic or carcinogenic (Black & Coon, 1987; Nebert & Gonzalez, 1987). In eukaryotes these enzymes are found bound to the membrane of the endoplasmic reticulum or mitochondrion.

In a recent review, the primary structures of 65 isoenzymes, from several species, were catalogued (Nebert et al., 1987). However, the three-dimensional structure of only one isoenzyme has been solved, that of cytochrome $P450_{cam}$, a soluble, bacterial protein (Poulos et al., 1985, 1987). Although the crystallization of bovine cytochrome $P450_{scc}$ has recently been reported (Iwamoto et al., 1988), the structure has yet to be solved. The primary structure of cytochrome $P450_{cam}$ shows only weak homology with the published sequences of the eukaryotic isoenzymes, except in several highly conserved regions, most notably in the area of the heme thiolate ligand (Poulos et al., 1987). Further structural information on the eukaryotic isoenzymes is difficult to obtain due to their membrane-bound nature.

In view of the wealth of information available on the sequences of these hemoproteins, it is pertinent to suggest ways in which this might be used to hypothesize upon the structure of the mammalian cytochromes P450 and to develop models upon which experimental work can proceed rationally. A number of such models have previously been proposed, using methods based upon either the local hydrophobicity of the

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