Separation and characterization of two polypeptide chains from the 7S cross-linking domain of basement-membrane (type IV) collagen

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(Received 15 March 1984/Accepted 21 May 1984)

The long-form 7S domain of human placental type IV collagen was prepared and after reduction, denaturation and aminoethylation, was separated into its subunits. The monomer subunit was further separated into two polypeptide chains of M_r about 25000. From compositional data and CNBr peptide patterns it was shown that the two chains were different. Furthermore, all subunits contained both chains, thus supporting a proposed subunit structure for the 7S domain and a chain composition $[\alpha 1(IV)]_2\alpha_2(IV)$ for the type IV molecule.

A macromolecular model for the structure of basement-membrane (type IV) collagen, which is based mainly on electron-micrographs of rotary-shadowed preparations, has been proposed (Kühn *et al.*, 1981). The basic structural unit is a tetramer of apparent M_r 2×10^6 . It is composed of four triple-helical collagen molecules joined at one end in a cross-linking domain referred to as the '7S domain'. The other ends of the molecules carry globular domains that are involved in the formation of a three-dimensional supramacromolecular network (Oberbäumer *et al.*, 1982). Within the 7S domain the four triple helices overlap one another by 30nm, which corresponds to a linear sequence of approx. 100 amino acids along the component polypeptide chains. The twelve chains in this domain are held together by disulphide cross-links and non-reducible cross-links. A large bacterial-collagenase-digested fragment of type IV collagen that contains the intact 7S domain has been described (Timpl *et al.*, 1979). The 7S domain of type IV collagen can be prepared in two different forms termed 'long' and 'short' (Kühn *et al.*, 1981). The long form, with an apparent M_r of 36×10^4 , contains four triple-helical molecules 60nm long that overlap for 30nm of their length. The short form contains only the overlapping region of the four component triple-helical molecules.

Studies on the biosynthetic products of type-IV-collagen-producing cells showed the presence of two distinct polypeptide chains in type IV collagen (Oberbäumer *et al.*, 1982; Alitalo *et al.*, 1980;

Tryggvason *et al.*, 1980). Fragments of type IV collagen, isolated from pepsin-solubilized basement-membrane-containing tissues, were also shown to be derived from two distinct polypeptide chains (Timpl *et al.*, 1979; Dixit, 1980; Glanville *et al.*, 1979; Gay & Miller, 1979). On the basis of a comparison of the chromatographic properties of denatured preparations of large pepsin-digested fragments of type IV collagen with reduced and denatured samples, it was suggested that the two chains were components of one triple-helical molecule (Trüeb *et al.*, 1982; Mayne *et al.*, 1982). Other evidence, from investigations of mouse tumour and type IV collagen (Timpl *et al.*, 1979; Robey & Martin, 1981), indicated that the chains are components of two homogeneous triple helices.

Here we present data derived from detailed structural investigations on the 7S cross-linking domain, which support a mixed-chain triple-helix $[\alpha 1(IV)]_2\alpha_2(IV)$ for type IV collagen, and indicate that both chains are involved in the formation of non-reducible crosslinks.

Methods

Solubilization of type IV collagen

Human placental tissue residue which had been minced and extracted once at 5°C with a 0.85% NaCl solution was supplied by Hoechst/Behringwerke, Marburg, Germany. The residue was washed with 0.4M-sodium acetate until free of blood and once with 0.5M-formic acid. The washed tissue (2 kg wet weight) was suspended in 6 litres of 0.5M-formic acid at 8–10°C (pH 2.0–2.1) and 1 g of pepsin was added (porcine pepsin; 2500 Anson units/mg; Boehringer, Mannheim). After stirring

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at 8–10°C for 16h, the digest was centrifuged and the solubilized type IV collagen purified as described previously (Glanville *et al.*, 1979).

Preparation of the 7S cross-linking domain of type IV collagen

To prepare the long form of the 7S domain, the type IV collagen fraction prepared as described above (2g) was dissolved in 30mM-Tris/HCl buffer, pH 7.6, containing 5mM-CaCl₂ and digested with bacterial collagenase (1.5mg; type IV clostridiopeptidase; Millipore Corp.) at 25°C for 24h. The digest was acidified, dialysed free of salt and freeze-dried. The average yield was 800mg.

The 7S domain was purified by chromatographing on a column (5cm × 125cm) of agarose A-5.0m equilibrated with 5mM-Tris/HCl buffer, pH 7.5, containing 1M-CaCl₂. From 2kg of wet placental tissue residue, 150–200mg of the purified long form of the 7S domain was obtained.

Reduction and aminoethylation

The 7S domain (100mg) was suspended in 10ml of water adjusted to pH 8.0 with NaOH and 100mg of NaBH₄ was added. The solution was left at room temperature for 1h, solid urea added to a final concentration of 8M and then the sample reduced by adding 100 μ l (1.44mol) of β -mercaptoethanol. The solution was incubated at 60°C for 1h and then at room temperature for 1h. The free thiol groups were aminoethylated by adding ethylenimine in three portions of 120, 60 and 100 μ l at 10-min intervals. After a further 30min at room temperature the solution was acidified and desalted on Bio-Gel P2 equilibrated with 0.01M-acetic acid, and freeze-dried. The extent of reduction and aminoethylation was checked by polyacrylamide-gel electrophoresis in 4.5% disc gels containing SDS (Weber & Osborn, 1969).

Separation of the subunits of the 7S domain

The reduced and aminoethylated 7S domain was chromatographed on a column (2.5cm × 140cm) of agarose A1.5m equilibrated in 5mM-Tris/HCl buffer, pH 7.5, containing 1M-CaCl₂. Fractions indicated in Fig. 1(a) were desalted, freeze-dried and applied to a reverse-phase high-pressure liquid-chromatography column (Vydac TP/C18; 10 μ m particle size; 250mm × 9mm; supplied by Chrompak, Middeburg, Holland). The column was equilibrated at 55°C with 0.1% trifluoroacetic acid containing 15% (v/v) acetonitrile at a flow rate of 4ml/min, and the sample was eluted with a linear gradient of acetonitrile to 65% in 45min. Pools collected were directly freeze-dried.

Characterization of the monomer fraction isolated from the 7S domain

Amino acid compositions of the two chains were determined on a Durrum (Palo Alto, CA, U.S.A.) D500 analyser. Samples were hydrolysed in 6M-HCl containing β -mercaptoethanol (10000:1, v/v), under N₂ at 110°C for 24h. No corrections were made for losses during hydrolysis.

Attempts to determine the *N*-terminal amino acid sequences of the chains before and after deblocking were made by using a liquid-phase automatic sequencer (Beckman; model 890B or 890C) (Babel & Glanville, 1984).

Attempts to deblock the *N*-terminus of the two isolated chains were made by using pyroglutamate aminopeptidase (4m-units/mg; Boehringer, Mannheim). The chains (200nm long) were dissolved in 65mM-sodium phosphate buffer, pH 7.4, and 1mg of enzyme was added. After 6h at 37°C the digest was dialysed free of salt and freeze-dried.

CNBr digests were carried out at 30°C for 4h in 70% formic acid. CNBr was removed under vacuum and the samples applied to a column (1.5cm × 140cm) of Sephadex G-75 (superfine grade) equilibrated in 50mM-ammonium acetate at 37°C.

Results

The inset in Fig. 1(a) shows purified 7S-domain long form, in the reduced and unreduced state. In the unreduced state a single band is seen with an apparent *M_r* of approx. 3.5×10^5 (Timpl *et al.*, 1981). After reduction, twelve bands are visible. The lower six bands, of apparent molecular sizes 25, 50, 75, 100, 120 and 140kDa, are of approximately equal intensity and much stronger than the upper six bands. Similar values for molecular size were calculated from the elution positions on calibrated molecular-sieve columns.

The chromatogram in Fig. 1(a) shows the separation of the 7S-domain subunits on a molecular sieve. The monomer fraction (25kDa) was further resolved into two components by using a reverse-phase high-pressure liquid-chromatography column as illustrated in Fig. 1(b). The weight of material recovered from the first peak was always less than from the second. On the basis of this observation, for working purposes, the chains are referred to as ' α 2(IV)25' and ' α 1(IV)25' respectively. This nomenclature does not imply necessarily that the α 1(IV)25 chain is related to the α 1(IV)95 chain sequenced recently (Babel & Glanville, 1984). The chain ratio in the monomer fraction may not reflect the true chain composition of the type IV collagen molecule, owing to the high proportion of cross-linked chains in the 7S domain. The dimer (50kDa) and trimer (75kDa)

Fig. 1. Preparation of $\alpha 1(IV)25$ and $\alpha 2(IV)25$ from the reduced and aminoethylated long-form 7S domain of type IV collagen (a) Separation of the subunits on agarose A1.5m. The arrow indicates V_0 and the bar marked 'I' the monomer fraction containing the $\alpha 1(IV)25$ and $\alpha 2(IV)25$ chains. The inset shows disc-electrophoresis gels of unreduced 7S long form on the left and reduced and aminoethylated 7S long form on the right. The smallest subunit in the reduced sample corresponds to the monomer fraction indicated by the bar marked 'I' in the chromatogram. (b) Separation of the $\alpha 1(IV)25$ and $\alpha 2(IV)25$ chains of the long-form 7S domain using high-pressure liquid chromatography. The monomer fraction indicated above was applied to a Vydac TP-C18 reverse-phase column and eluted with a linear gradient of acetonitrile. The first component to be eluted was the $\alpha 2(IV)25$ chain and the second $\alpha 1(IV)25$. Fractions were pooled as indicated by the bars.

fractions from Fig. 1(a) could not be further resolved by rechromatographing them on reverse-phase high-pressure liquid-chromatography columns (results not shown).

The amino acid compositions of the intact long form of the 7S domain and its constituent α -chains are shown in Table 1. Significantly higher values for valine, tyrosine and arginine, and significantly

Table 1. Amino acid composition of long-form 7S and its component α -chains

Amino acid	Composition (mol/1000mol)		
	7S	$\alpha 1(IV)25$	$\alpha 2(IV)25$
Hydroxyproline	127	145	73
Aspartic acid	50	46	48
Threonine	24	30	17
Serine	19	23	26
Glutamic acid	100	90	100
Proline	86	90	90
Glycine	305	305	306
Alanine	21	22	28
Cysteine	10	13	10
Valine	34	24	39
Methionine	8	12	3
Isoleucine	18	24	8
Leucine	53	63	25
Tyrosine	12	6	20
Phenylalanine	26	25	24
Histidine	10	11	8
Hydroxylysine	42	52	39
Lysine	7	6	9
Arginine	35	25	50

lower values for hydroxyproline, threonine, methionine and isoleucine, were found in the $\alpha 2(IV)25$ chain when compared with the $\alpha 1(IV)25$ chain. In general the $\alpha 1(IV)25$ chain has a higher content of hydrophobic amino acids and the $\alpha 2(IV)25$ chain a higher content of the charged polar amino acids. Of particular interest was the low methionine content of the $\alpha 2(IV)25$ chain, which corresponds to only one residue per molecule. It appeared, therefore, on the basis of amino acid compositions, that the type IV 7S domain contained two different polypeptide chains.

To confirm this, the $\alpha 1(IV)25$ and $\alpha 2(IV)25$ chains were cleaved with CNBr and the peptides separated on Sephadex G-75 (superfine grade) as shown in Figs. 2(a) and 2(b) respectively. Clearly the elution patterns are different. In Fig. 2(c) the elution profile from Fig. 2(a) is compared with that of a CNBr digest of the purified trimeric subunit of the 7S domain. Between the elution volumes of 328 and 345 ml the profiles are practically identical, indicating the presence of $\alpha 1(IV)25$ in the trimer. Between 345 and 350 ml, a peak in the trimer profile is present which corresponds to the lowest- M_r component in Fig. 2(b). Therefore the trimer also contains the $\alpha 2(IV)25$ chain. The large peak eluting between 310 and 320 ml presumably contains peptides cross-linked by lysine-derived tri-functional cross-links. The cross-linked peptides appear to originate from the region between elution volumes 318 to 328 ml in Figs. 2(a) and 2(b).

Fig. 2. Elution profiles of the CNBr peptides of (a) $\alpha 1(IV)25$, (b) $\alpha 2(IV)25$ and (c) the trimeric subunit (curve ii) and the $\alpha 1(IV)25$ chain (curve i) chromatographed on Sephadex G-75 (superfine grade)

The arrows indicate V_0 . Note the difference between the patterns in (a) and (b) and the similarity of the two curves in (c). The peak marked with a bar in (b) is also present in the trimer chromatogram in (c), also marked with a bar, indicating the presence of $\alpha 2(IV)25$ in the trimer.

Discussion

In the preparation procedures described here for the long form of the type IV collagen 7S domain, the conditions for the collagenase digest are critical. Incomplete cleavage leads to a heterogeneous product in which the 7S domain carries arms of various lengths from 30 nm to over 300 nm. This material, when reduced, is visible on polyacrylamide-disc-gel electrophoretograms as bands with M_r values of more than 150 000 or bands that run between the expected six major components. Conversely, the use of more intensive cleavage conditions leads to a mixture of long and short forms of the 7S domain that are not easily detected by disc gel electrophoresis, since the bands of reduced long- and short-form 7S, with the exception of the monomer components, which do not

stain very intensely, practically coincide. The best method to control the homogeneity of a preparation is to prepare electron-micrographs of samples prepared by using the rotary-shadowing technique (Kühn *et al.*, 1981). It was found advantageous to treat the 7S domain first with NaHB_4 to reduce any lysine aldehyde groups that may be present. This treatment also increased the yield of the monomer fraction. After reducing and aminoethylating the disulphide bridges of the 7S domain and isolating the resulting fragments, it was found that only the lowest- M_r fraction could be resolved into two components by high-pressure liquid chromatography. The CNBr peptide patterns of all fractions were, with the exception of those regions containing cross-linked peptides, identical. Furthermore, peptides from both the $\alpha 1$ - and $\alpha 2$ -chains were present in all fractions. This, taken together with the apparent M_r values for the fragments, indicate a monomer/dimer/trimer/tetramer/pentamer/hexamer subunit relationship for the six major components, each of which contains both $\alpha 1$ - and $\alpha 2$ -chains cross-linked in all but the monomer fraction. These results therefore confirm a previously proposed subunit structure for the 7S domain (Timpl *et al.*, 1981). The presence of six major and six minor subunits (see the insert to Fig. 1) representing monomers to dodecamers suggest a complex cross-linking arrangement, probably involving polyfunctional cross-links such as pyridinoline (Robins & Duncan, 1983). Only about 10–15% of the chains are not cross-linked, and dimers to hexamers appear to be present in substantially higher concentrations than the higher oligomers. Preliminary experiments have shown the presence of a novel non-reducible cross-link that is an Ehrlich chromogen (Scott *et al.*, 1983), lending support to the proposed function of the 7S domain as a cross-linking region. The presence of two different chains in the preparations of the 7S domain described here can be interpreted in three different ways. It is possible that the preparations contained two different 7S domains, one containing 12 $\alpha 1$ -chains and another 12 $\alpha 2$ -chains, which were purified together. This is unlikely, since one would expect to be able to separate such homopolymer molecules, or subunits from them, by high-pressure liquid chromatography, in a similar way to the monomer chains. This was not possible (results not shown). In addition, because of the different proteinase-susceptibilities of the $\alpha 1$ - and $\alpha 2$ -chains (R. W. Glanville, R. Qian & B. Siebold, unpublished work), one would not expect the 7S domains, composed of different chains, to have the same molecular dimensions. Preparations of long-form 7S under the electron microscope have very uniform molecular dimensions (Kühn *et al.*, 1981). The second possibility is to have a 7S

domain in which two triple helices contain only $\alpha 1$ -chains and two only $\alpha 2$ -chains. However, from electron-micrographs it is known that all four arms of the type IV molecule have the same chain composition, because of the symmetry of the intact molecule and of the long-form 7S domain. Therefore the most likely arrangement is four triple-helical molecules each with a chain composition $[\alpha 1(\text{IV})]_2\alpha 2(\text{IV})$. This supports the findings of others (Trüeb *et al.*, 1982; Mayne *et al.*, 1982) and further substantiates the proposed network model for basement-membrane collagen (Oberbäumer *et al.*, 1982).

Attempts to determine the *N*-terminal amino acid sequence of the isolated monomer chains of the long form of the 7S domain failed. As the *N*-terminal ends of the 12 chains of type IV collagen are contained in the 7S domain (Dixit *et al.*, 1983; Weber *et al.*, 1984; Fessler & Fessler, 1982), it may be that the *N*-terminal ends of both the $\alpha 1$ - and $\alpha 2$ -chains are blocked. This would be analogous to the situation in interstitial collagens (types I, II and III), where all intact α -chains have an *N*-terminal pyroglutamic acid residue. However, pepsin treatment of interstitial collagens removes an *N*-terminal non-helical peptide, thus deblocking the α -chains. This does not appear to happen in the 7S domain. Attempts to deblock the isolated chains with the enzyme pyroglutamate aminopeptidase failed.

We thank Professor K. Kühn for his helpful discussions and guidance, Miss Sabine Rannenber for running the amino acid sequencers, Mr. Wolfgang Strasshofer for running the amino acid analysers and Mr. Albert Ries and Mrs. Veronika Legner for technical assistance. This research was supported in part by grants from the Deutsche Forschungsgemeinschaft (G1 106/1-1).

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