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Identification of the Sites in Collagen α-Chains that Bind Serum Anti-Gelatin Factor (Cold-Insoluble Globulin)

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Anti-gelatin factor was prepared from guinea-pig and human serum by affinity chromatography on denatured type-I collagen. As shown previously, this component is related to cold-insoluble globulin. It reacted with ^{125}I-labelled denatured collagen, and the reaction could be inhibited by preincubation with unlabelled collagenous components. In the inhibition assay comparable activities were observed for native and denatured type-I, -II, -III and -IV collagens. There was also no difference in reactivity between collagens of different species. The reactive sites in the collagen α-chains were located by inhibition assays on distinct CNBr- and collagenase-derived peptides. The results obtained with fragments from α1(I)-, α2- and α1(II)-chains indicate that the most active region is located between positions 643 and 819 of the α1-chain. Lower activities were found for other regions of collagen and may indicate that the factor has the potential to interact with several sites in the α-chains. The present data agree with observations by Kleinman, McGoodwin & Klebe [*Biochem. Biophys. Res. Commun.* (1976) **72**, 426–432] on the specificity of a serum factor promoting the attachment of fibroblasts to collagen.

Serum from a variety of mammalian species contains a protein, 'anti-gelatin factor', which binds to denatured collagen (Maurer, 1954*a*,*b*, Wolff *et al.*, 1967). Anti-gelatin factor can be detected by passive haemagglutination or radioimmunoassays (Wolff *et al.*, 1967; Bray *et al.*, 1969; Adelmann *et al.*, 1973; Nowack *et al.*, 1975; Hopper *et al.*, 1976). It has been found that anti-gelatin factor is similar to, or even identical with, cold-insoluble globulin, as judged by its electrophoretic mobility, amino acid composition, identity on immunodiffusion and capacity to mediate uptake of denatured collagen by guinea-pig peritoneal-exudate cells (W. Dessau, F. Jilek, B. C. Adelmann & H. Hörmann, unpublished work).

Cold-insoluble globulin has also been shown to resemble fibronectin, a high-molecular-weight protein produced by fibroblasts (Vaheri *et al.*, 1977), as well as several other surface-associated proteins (Hynes & Humphryes, 1974; Hogg, 1974; Yamada & Weston, 1974; Rouslahti & Vaheri, 1975; Pearlstein, 1976). Since the identity of these various proteins has not yet been completely established, in the present paper we refer to the protein that we have studied as 'anti-gelatin factor'.

Klebe (1974) has described a protein in serum, 'cell-attachment protein', required for the binding of fibroblasts to collagen matrices. Jilek & Hörmann (1977) have recently shown that 'cell-attachment protein' and 'cold-insoluble globulin' are related proteins. Kleinman *et al.* (1976) investigated the specificity of this interaction by locating the binding site(s) in the chains of collagen. Peptides derived from only a certain region of the α1(I)-chain were able to bind to 'cell-attachment protein', suggesting that binding involved a specific site on the collagen molecule. Here we have carried out similar studies to delineate the interaction of anti-gelatin factor and collagen and to locate the binding site.

Materials and Methods

Purification of anti-gelatin factor

Gelatin-binding proteins were isolated from 150ml of human or guinea-pig serum. The serum was passed at 4°C through a column of denatured type I-collagen (50mg) coupled covalently to CNBr-activated Sepharose 4B (50ml). The column was washed with 750ml of 0.05M-Tris/HCl (pH7.6)/0.1M-NaCl/0.04M-ε-aminohexanoic acid. Bound material was then eluted from the column with 1M-KBr/0.05M-Tris/HCl (pH5.3)/0.025M-ε-aminohexanoic acid. The eluate was dialysed against 0.1M-Tris/HCl (pH7.4)/0.1M-NaCl and concentrated by ultrafiltration (Diaflo). The A_{280} of the concentrated solution was 1.5, corresponding to a concentration of about 0.9mg of anti-gelatin factor/μl.

Preparation of collagens, α-chains and peptides

Type-I collagen was purified from an acidic extract of calf skin (Pontz *et al.*, 1970). Limited digestion with

pepsin (Miller, 1972) was used to solubilize type-II collagen from bovine articular cartilage. Type-III collagen was purified from a pepsin digest of calf skin (Fujii & Kühn, 1975). Type-IV collagen was extracted and purified from a mouse tumour producing an extracellular matrix of basement membrane (Orkin *et al.*, 1977). Denatured collagens were obtained by heating a solution (0.5mg/ml) in 0.05% acetic acid for 60min at 56°C. The α1(I)- and α2-chains of type-I collagen were separated by chromatography on CM-cellulose (Piez *et al.*, 1963).

CNBr peptides were prepared from rabbit collagen α1(I)-chain (Becker & Timpl, 1972), from rat collagen α2-chain (Fietzek & Piez, 1969) and from bovine α1(II)-chain (Miller & Lunde, 1973) by established procedures. The peptides of α1(II)-chain used in this study were kindly supplied by Dr. H. Kleinman and Dr. E. J. Miller. Additionally, we prepared a 78nm-long triple-helical fragment of type-I collagen by limited digestion with bacterial collagenase. The peptides comprising this fragment, α1(I)-C(78) and α2-C(78), were purified as described previously (von der Mark *et al.*, 1970).

The purity of these materials was judged by amino acid analysis and by polyacrylamide-gel disc electrophoresis in sodium dodecyl sulphate-containing buffers (Furthmayr & Timpl, 1971).

Antisera

Purified anti-gelatin factor from either human or guinea-pig serum (150μg/0.5ml of 0.05M-Tris/HCl buffer) was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into goats. This injection was repeated each week for 5 weeks. Blood was collected from the goats starting 1 week after the last injection. Before use, antisera (150ml) were passed through a column of denatured collagen (50mg) to remove goat serum anti-gelatin factor.

Radioassay for anti-gelatin factor

Native bovine type-I collagen was labelled with ^{125}I (NEN, Dreieichenhain, W. Germany) by the chloramine-T method as described previously (Adelmann *et al.*, 1973). Before the assay, collagen was denatured by heating at 56°C for 60min. Binding of denatured collagen by anti-gelatin factor was determined in duplicate tubes containing 0.1ml of anti-gelatin factor at different dilutions, 0.1ml of denatured ^{125}I-labelled collagen (100ng) and 0.2ml of 0.1M-Tris/HCl (pH7.4)/0.1M-NaCl/0.1% bovine serum albumin. After incubation for 12h at 4°C, goat antiserum (0.2ml) to anti-gelatin factor was added and the mixture further incubated for 24h at 4°C. Precipitated material was collected by centrifugation (2000*g* for 30min) and washed with 3×0.6ml of 0.1M-Tris/HCl (pH7.4)/0.1M-NaCl/0.1% bovine serum albumin. Radioactivity of the precipitates was determined in a scintillation spectrometer (Gamma 300, Beckman).

To assess non-specific precipitation, similar reaction mixtures were prepared but lacking anti-gelatin factor. These are referred to in the formula listed below as 'non-specific binding'. The amount of radioactivity precipitated under these conditions was 5–8%.

Quantitative assessment of the binding of denatured collagen to anti-gelatin factor was calculated by a standard procedure used in radioimmunoassays (Minden & Farr, 1973).

Inhibition assay

After assay of preparation of anti-gelatin factor, sufficient anti-gelatin factor to bind 35–40% of the labelled collagen was preincubated for 12h at 4°C with various amounts of native collagen, α-chains and peptides to be tested for activity in 0.1M-Tris/HCl (pH7.4)/0.1M-NaCl/0.1% bovine serum albumin. After addition of the labelled collagen, the procedure outlined in the preceding paragraph was followed. Components that react with anti-gelatin factor inhibit subsequent precipitation of labelled collagen. The results were expressed as:

$$\%\ \text{inhibition} = \frac{(\text{anti-gelatin factor}+\text{inhibitor})\text{c.p.m.} - (\text{non-specific binding})\text{c.p.m.}}{(\text{anti-gelatin factor})\text{c.p.m.} - (\text{non-specific binding})\text{c.p.m.}} \times 100$$

The molar amount of material that would produce a 50% inhibition was calculated from a semilogarithmic plot of inhibitor concentration versus percentage inhibition. In calculating molar equivalents a mol.wt. of 95000 was assumed for native and denatured collagen and for α-chains. The molecular weights assumed for collagen peptides were those reported previously (Fietzek & Piez, 1969; von der Mark *et al.*, 1970; Becker & Timpl, 1972; Miller & Lunde, 1973).

Results

The precipitation of denatured ^{125}I-labelled collagen in the presence of different amounts of anti-gelatin factor is shown in Fig. 1(*a*). A maximum of 70% of the radioactivity was precipitated, suggesting that a substantial portion of the label was associated with material that reacted in the assay. Similar curves were obtained when denatured type-II and type-III collagens were substituted for type-I (results not shown).

The precipitation of the labelled collagen was

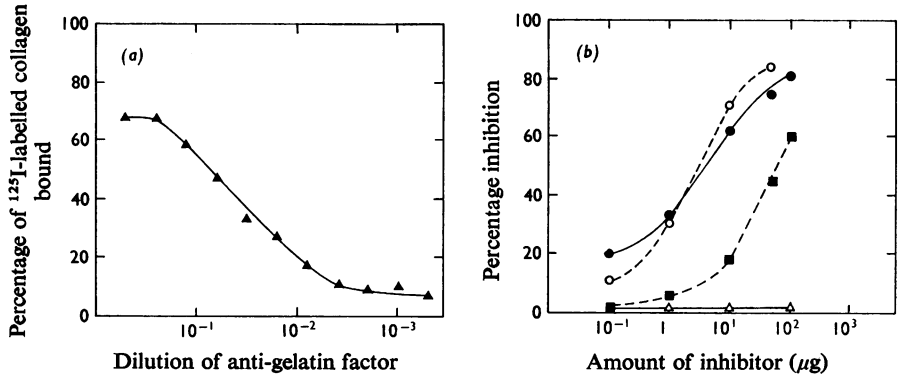

Fig. 1. *Binding of ^{125}I-labelled denatured bovine collagen by human anti-gelatin factor (a) and inhibition of binding by various collagen peptides (b)*

In both assays bovine type-I collagen (100ng) was used as labelled component. All inhibitors were from rabbit type-I collagen. Complexes between collagen and anti-gelatin factor were separated from non-bound collagen by precipitation with antibodies to anti-gelatin factor. ▲, Peptide α1(I)-CB2; ■, peptide α1(I)-CB3; ●, α1(I)-chain; ○, peptide α1(I)-CB7.

Table 1. *Inhibitory activity of native and denatured collagens for anti-gelatin factor*

In all tests ^{125}I-labelled denatured type-I collagen from calf was used. n.d., Not determined.

Inhibitors	Source of inhibitor	Concentrations required for 50% inhibition of anti-gelatin factor (nM)	
		Guinea pig	Human
Type-I collagen, native	Calf	0.018	0.018
Type-II collagen, native	Calf	0.186	0.420
Type-III collagen, native	Calf	0.021	0.018
Type-IV collagen, native	Mouse	n.d.	0.021
Type-I collagen, denatured	Calf	0.047	0.011
Type-II collagen, denatured	Calf	0.019	0.042
Type-III collagen, denatured	Calf	0.053	0.011
α1(I)-chain	Rat	0.053	n.d.
α1(I)-chain	Rabbit	0.037	0.042
α2-chain	Calf	0.037	0.037
α1(II)-chain	Rat	0.037	n.d.

inhibited when certain unlabelled collagenous components were preincubated with anti-gelatin factor. A semilogarithmic plot of concentration versus percentage inhibition was approximately linear in the range between 20 and 80% inhibition and resembled in form the curves obtained in radioimmunoassays when inhibitors are used.

Type-III collagen was as active as type-I collagen in blocking the reaction, but type-II collagen was less active. However, after denaturation the three proteins were equally active. Strong inhibitory activity was also observed for native type-IV collagen from basement membrane. Activity was retained after chromatographic purification, and equal activity was exhibited by the α1(I)- and α2-chains obtained from type-I collagen. No differences were noted when bovine, rat and rabbit chains were tested (Table 1).

The ability of various peptides prepared by CNBr digestion of the α1(I)-, α2- and α1(II)-chains was evaluated. As shown in Fig. 1(*b*), peptide α1(I)-CB7 was as effective an inhibitor as the α1(I)-chain, α1(I)-CB3 peptide was less active and α1(I)-CB2 peptide was inactive. Analysis of the results obtained in such assays (Table 2) indicated that peptides α1(I)-CB7 and α2-CB5 showed activity similar to their parent chains, whereas other peptides were either inactive or less active. However, the collagenase-produced fragments α1(I)-C(78) and α2-C(78) had activity comparable with peptide α1(I)-CB7. Four of the CNBr peptides derived from the α1(II)-chain were also examined and the most active was α1(II)-CB10.

In these studies, anti-gelatin factor from both guinea-pig and human serum was used. Similar

Table 2. *Inhibition of the reaction between anti-gelatin factor and denatured collagen by CNBr peptides of collagen α-chains*
The ^{125}I-labelled collagen was type-I in the tests with α1(I)- and α2-peptides and type-II in the tests with α1(II)-peptides. n.d., Not determined.

Inhibitors	Concentration required for 50% inhibition of anti-gelatin factor (nM)		Concentration required to inhibit attachment of cells to collagen (nM)*
	Guinea pig	Human	
α1(I)-chain	0.04	0.04	4
Peptides: α1(I)-CB1 or α1(I)-CB2	>50	>50	Inactive
α1(I)-CB3	2.63	4.74	⩾300
α1(I)-CB4 or α1(I)-CB5	>23	>23	⩾1000
α1(I)-CB6A	17.93	2.25	⩾200
α1(I)-CB7	0.06	0.13	21
α1(I)-CB8	0.58	0.26	83
α1(I)-C(78)	0.08	0.08	n.d.
α2-chain	0.04	0.04	17
Peptides: α2-CB1 or α2-CB2	>37	>37	n.d.
α2-CB3	0.94	0.24	n.d.
α2-CB4	1.07	0.33	n.d.
α2-CB5	0.09	0.03	n.d.
α2-C(78)	0.14	0.03	n.d.
α1(II)-chain	0.04	n.d.	7
Peptides: α1(II)-CB4+6	1.33	1.33	⩾1000
α1(II)-CB8	0.65	0.29	⩾300
α1(II)-CB10	0.09	0.24	31
α1(II)-CB11	0.62	0.28	

* Data taken from Kleinman *et al.* (1976).

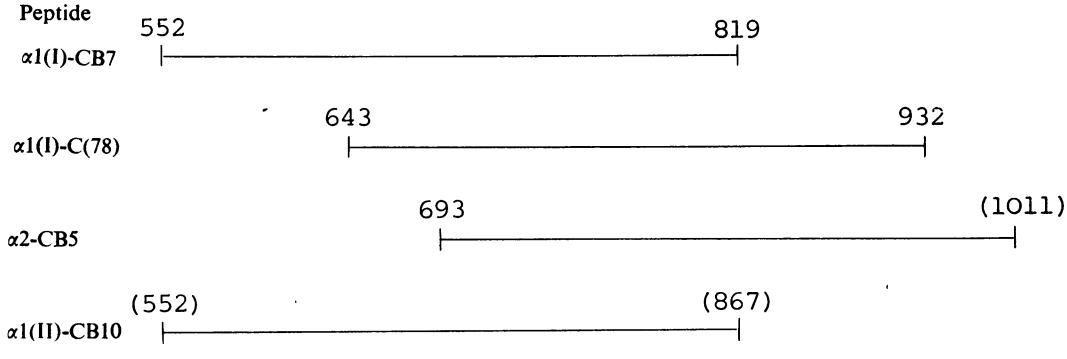

Fig. 2. *Location in collagen α-chain of peptides that show highest activity in inhibiting the reaction of denatured ^{125}I-labelled collagen and anti-gelatin factor*
The sequence data are taken from Fietzek & Kühn (1976). The numbers refer to the positions of the *N*-terminal and *C*-terminal amino acids of each peptide. Numbers in parentheses are tentative.

results were obtained with both preparations (Tables 1 and 2), except that human anti-gelatin factor showed equal reactivity for three of the four CNBr-derived peptides of α1(II)-chain.

Discussion

As shown previously (Adelmann *et al.*, 1973; Nowack *et al.*, 1975; Hopper *et al.*, 1976) the reaction of denatured collagen with anti-gelatin factor can be detected by a radioassay. The specificity of this reaction was assessed in the present study by inhibition assays.

Strong inhibiting activity was observed for triple-helical and denatured collagen, indicating that under our assay conditions the conformation of collagen is not important for this reaction. Comparable activity was also found for genetically distinct types of

collagens. Type-I, -II and -III collagens have different chain compositions, i.e. $[\alpha 1(I)]_2\alpha 2$, $[\alpha 1(II)]_3$ and $[\alpha 1(III)]_3$ (Miller & Matukas, 1974). However, homologous sequences exist in these α-chains (Fietzek & Kühn, 1976). Corroborating the results of Wolff & Timpl (1968), anti-gelatin factor did not distinguish between collagens obtained from different species. These observations indicate that the collagen sequences involved in the reaction are not highly mutable.

The location of the reactive sites in the collagen α-chains is shown in Fig. 2. Since the overlapping peptides α1(I)-CB7 and α1(I)-C(78) show equal activity, it is likely that the most active binding region of the α1(I)-chain is located between positions 643 and 819 of the sequence (Fietzek & Kühn, 1976). Homologous portions may be involved in α2- and α1(II)-chains. The single peptide bond cleaved by animal collagenase (Gross, 1976) is also contained in this region. Previous studies with different types of α-chains have established that the sequences around the cleavage site show a highly conservative structure (Fietzek & Kühn, 1976; Miller *et al.*, 1976). It is possible, however, that other portions in the α-chain participate in the reaction with anti-gelatin factor. For example, distinct activity was also found on peptide α1(I)-CB8, which occupies positions 124–402 in the α1(I)-chain (Fietzek & Kühn, 1976).

Our data on the specific regions of the α1(I)-, α2- and α1(II)-chains that react with anti-gelatin factor resemble the results obtained by Kleinman *et al.* (1976) with the collagen-attachment protein from serum (cf. last column in Table 2). The major difference is that they found α2-chain to be significantly less active than α1(I). However, this could reflect differences in the conditions used to assay the serum factors, since they incubated peptides with cell-attachment factor for less time.

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