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# Platelet-reactive sites in collagen

## Collagens I and III possess different aggregatory sites

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Collagen type III possesses a highly reactive platelet-aggregatory site at a locus which in type I is essentially inactive whilst the latter collagen possesses reactive sites absent in type III. It is proposed that platelet aggregation by collagen involves the sequence GK[or R]PG(EY)GPK[or R]G(EY) or, less favourably, GPK[or R]G(EY)G(XY)GK[or R]PG(EY), one basic residue acting in combination with the second in an adjacent α-chain.

## INTRODUCTION

Collagens in the blood vessel wall (and perivascular space) fulfil an essential function in haemostasis by causing the aggregation of blood platelets with which they come in contact following vessel injury [1,2]. Associated with this, they may be an important factor in the initiation of thrombosis, the pathological equivalent of the haemostatic process. This may be especially the case in thrombus formation associated with atherosclerotic lesions in the vessel wall. This is regarded as a serious complication of atherosclerosis, very probably the cause of sudden death from heart failure. It is thought to occur when an atherosclerotic plaque fissures [3], an event likely to lead to the exposure of thrombogenic elements, particularly collagens, within the plaque to circulating platelets.

The main collagenous species of the vessel wall are the two interstitial collagens, types I and III, fibres of which exhibit a very marked platelet-aggregatory activity [2]. In previous studies, we have established the presence of platelet-reactive sites in both the α1(I)- and α2(I)-chains of type I collagen [4], in the *N*- and *C*-terminal portions of the molecule and in at least four reactive sites in the α1(I)-chain [5]. We concluded that type I collagen appeared to contain a number of platelet-aggregatory sites of differing reactivity, distributed along the length of the molecule; individual sites we considered to be of relatively low affinity and perhaps, then, of relatively low structural specificity in terms of their actual amino acid sequence. Each site was thought to involve two (or more) basic residues, probably lysines, located in adjacent α-chains and the specific orientation of one to the other, crucial for activity, being dictated by collagen's triple-helical structure.

In the present study we have attempted to establish the location of platelet-reactive sites in collagen type III. Our results indicate a very different distribution to that in type I. Comparison of the reactivity of type I-derived peptides with the equivalent peptides from collagen type III has enabled us to tentatively propose a more detailed structure for platelet-aggregatory sites in collagens.

A preliminary account of some of this work has been given already [6].

## MATERIALS AND METHODS

### Collagens

Collagen type I was obtained as previously [4,5] from calf skin by extraction with sodium citrate buffer, pH 3.7 [7]. Type III collagen was obtained from the same source by pepsin digestion and subsequent precipitation with NaCl at acid and then neutral pH [8]. Collagen type-I α1(I)-chains were isolated as previously [4], and similarly, γ-chains of type III collagen [comprising three α1(III)-chains covalently linked by disulphide bonds] by chromatography on CM-cellulose [9].

Native-type fibres of collagen type I from bovine tendon were received as a finely dispersed suspension (10 mg/ml) generously donated by Ethicon Inc., Somerville, NJ, U.S.A. and details of which have been previously given [10]. Prior to use, a sample of the suspension was diluted to the required concentration with 0.01 M-acetic acid and dialysed against the same. The preparation remained finely-dispersed during this procedure.

### Isolation of peptides obtained by cleavage with CNBr

Collagen type I-derived peptides, α1(I)CB3 and CB7, were obtained from the α1(I)-chain as described previously [5]. Collagen type III α-chains (as γ-chains) were digested with CNBr and the resultant peptides separated on CM-cellulose as described by Rauterberg *et al.* [11]. The peptides α1(III)CB4 and α1(III)CB5 were further purified by rechromatography on CM-cellulose, and peptides α1(III)CB1,8,10,2 and α1(III)CB9A by gel filtration [11]. Homogeneity of peptide preparations was established by SDS/polyacrylamide-gel electrophoresis [12] using 12.5% (w/v) acrylamide gels.

### Renaturation

Following isolation, peptides from collagen α1(I)- and α1(III)-chains were renatured to restore triple-helical configuration, as we described previously [5], by stepwise cooling in 0.225 M-sodium citrate buffer, pH 3.7 [7]. The renaturation of collagen type-III γ-chains was undertaken precisely as we described earlier for collagen type-I α-chains [4].

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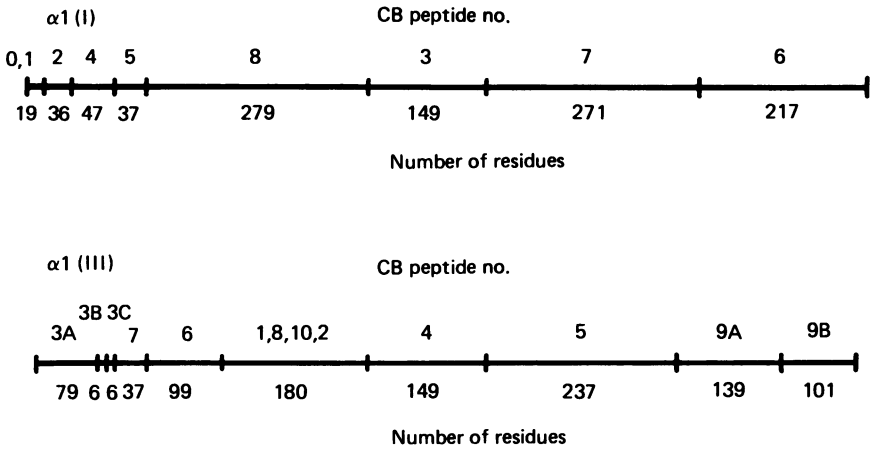


**Fig. 1. Diagram to illustrate the cleavage by CNBr of the α1(I)-chain of type I and the α1(III)-chain of type III bovine collagens**

### Polymerization

Collagen molecules, or molecules of collagen-derived renatured peptides, were polymerized by random molecular association by cross-linking with glutaraldehyde as in previous studies [5], using the conditions of Santoro & Cunningham [13]. We have observed, in agreement with these authors, that a concentration of cross-linking reagent in the region of 0.25% is the optimum to yield the polymer with the highest platelet-aggregatory activity and this is the concentration adopted in the present study. Under these conditions, we find, as do Santoro & Cunningham [13], that approx. 40% of lysyl residues have reacted with glutaraldehyde.

Native-type fibres of collagen type III were produced by prolonged dialysis of a solution of the collagen at 4 °C against *I* 0.02 sodium phosphate buffer, pH 7.6.

### Amino acid modification reactions

Arginyl residues in collagen were modified by treatment of a suspension of collagen fibres, or of collagen-derived renatured peptides, cross-linked by glutaraldehyde to an insoluble polymer, with phenylglyoxal, as described by Jorgensen *et al.* [14] for antithrombin III, using the reagent at a concentration of 40 mM and a reaction time of 2 h. After this time, the reaction was terminated by dilution with 5 vol. of 0.2 M-Tris/HCl buffer, pH 7.4, containing 0.1 M-NaCl at 0 °C. Samples were then collected by centrifugation and washed with cold, deionized water.

Lysyl residues in similar preparations were modified by treatment with trinitrobenzenesulphonic acid using the conditions of Wilner *et al.* [15] for insoluble human collagen, with a reaction time of 2 h. After reaction, samples were recovered by centrifugation and washed with cold, deionized water.

In all modification experiments, comparison was made with a control exposed to precisely the same reaction conditions as the test sample except for the omission of the specific modification reagent.

### Platelet aggregation

Aggregation of platelets was measured photometrically in an aggregometer with recorder, using human citrated platelet-rich plasma, as described in detail previously [5].

Inhibition of aggregation was assessed by preincubation of the platelet suspension with the test sample, 5 min prior to the addition of collagen fibres used to induce aggregation. These were added in an amount just sufficient on its own to cause a normal aggregatory response.

## RESULTS

In view of the importance of collagen tertiary and quaternary structure for platelet aggregatory activity, peptides in this (as in our previous) study [5] were first renatured to restore collagen triple-helical conformation and then polymerized to impose quaternary form, prior to testing their platelet reactivity. Platelet aggregation was measured at a temperature low enough (routinely 20 °C) to ensure retention of tertiary structure.

The peptide α1(III)CB4 from type III collagen (see Fig. 1) proved to be extremely active towards platelets. Several preparations were tested and found in each case to be able to cause an aggregation of platelets at a minimum concentration of around 0.5 μg/ml or less (Fig. 2). The parent collagen (i.e. as obtained by renaturation of isolated type-III γ-chains), similarly polymerized, induced an aggregation at concentrations of 10–20 μg/ml and greater. The activity of peptide α1(III)CB4 compares very favourably with that of native-type fibres of collagen (type I or III; see [16]). Thus our standard collagen (type I) fibre preparation from bovine tendon routinely caused platelet aggregation at a minimum concentration of approx. 0.5 μg/ml at 37 °C or, when measured at 20 °C, of around 1 μg/ml. Reconstituted type III fibres were active at a minimum concentration (at 37 °C) of around 5 μg/ml.

The type I peptide α1(I)CB3 (Fig. 1), renatured and cross-linked at the same time as peptide α1(III)CB4, failed to cause platelet aggregation when tested at concentrations up to 1 mg/ml (see Fig. 2), despite its close similarity in structure to α1(III)CB4. Peptide α1(I)CB3 had previously been found by us to be of relatively low platelet-aggregatory activity, only causing aggregation at a concentration of 200 μg/ml or over [5].

In our previous work, peptide α1(I)CB7 was the most active of the type I peptides examined [5], able to cause

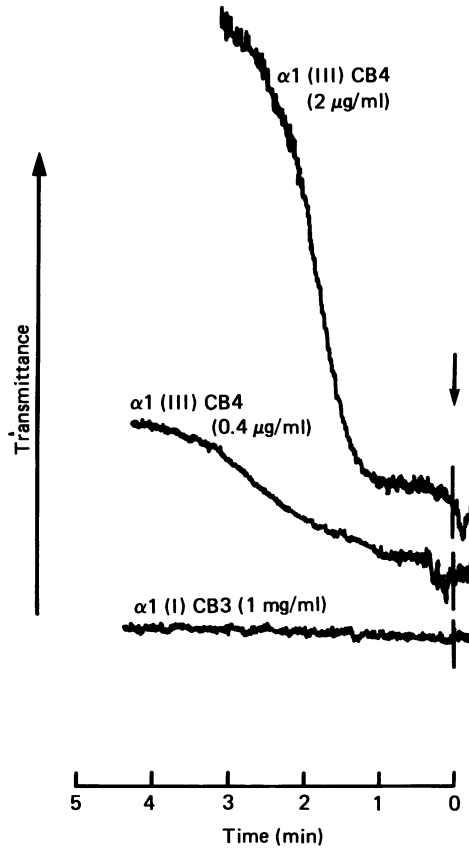


**Fig. 2. Platelet reactivity of the peptides α1(I)CB3 and α1(III)CB4**

Platelet aggregation, measured at 20 °C in a platelet aggregometer using human citrated platelet-rich plasma [5], is registered as an increase in light transmittance. Peptides, prior to testing at the concentrations indicated, were first renatured and then polymerized as described in the Materials and methods section. The arrow indicates the point of addition of sample.

aggregation at 5 μg/ml (or higher). In the current study, this peptide again exhibited appreciable reactivity, the minimum concentration required for aggregation being in the range 2.5–20 μg/ml. In contrast, the equivalent type III peptide, α1(III)CB5 (Fig. 1), renatured and polymerized alongside α1(I)CB7, was largely inactive, occasionally exhibiting a low activity (requiring for detection a minimum concentration of 1 mg/ml or more). Peptides α1(III)CB1,8,10,2 and α1(III)CB9A from type III collagen were also inactive or sometimes just so when tested at concentrations up to 1 mg/ml.

Neither α1(III)CB4 nor α1(III)CB5, when tested up to concentrations of 1 mg/ml as the free peptide lacking tertiary and quaternary form, was able to inhibit platelet aggregation induced by collagen type-I fibres (from bovine tendon) or by glutaraldehyde-cross-linked type-III collagen.

### Effect of chemical modification of lysyl and arginyl residues in reactive peptides

Evidence has been presented for the importance of collagen lysyl [15,17] and arginyl [18] residues in collagen-induced platelet aggregation. We decided therefore, to examine the effect of trinitrophenylation of lysyl residues, and modification of arginyl residues with phenylglyoxal, on the activity of the two peptides α1(III)CB4 and α1(I)CB7 and the parent collagens. We found that trinitrophenylation of our standard type-I fibre preparation (from bovine tendon) under conditions known to modify all lysyl residues [15], caused a loss of platelet aggregatory activity of 80–90%, in accord with the results of Wilner *et al.* [15]. Although reaction with phenylglyoxal had little direct discernable effect on the activity of the fibres, it did cause the loss of the residual activity remaining after treatment of the fibres with trinitrobenzenesulphonic acid. In the case of collagen type-III fibres (reconstituted *in vitro* as described), trinitrophenylation caused almost total loss of activity (95–100%).

Treatment of the peptide α1(III)CB4 (renatured and cross-linked) with phenylglyoxal had no effect on its ability to cause platelet aggregation, but trinitrophenylation caused a large loss (over 95%) of activity. In contrast, trinitrophenylation of α1(I)CB7 was totally without effect whilst treatment with phenylglyoxal reduced activity by around one half (see Fig. 3).

## DISCUSSION

The four collagen type-III-derived peptides α1(III)-CB1,8,10,2, CB4, CB5 and CB9A account together for approx. 70% of the total length of the α1(III) chain (Fig. 1). One of these peptides, α1(III)CB4, proved to be extremely active towards platelets, very much more reactive than the parent collagen (similarly polymerized) and the most reactive collagen-related species we have so far encountered. The remaining peptides were to all intents and purposes inactive. Our results suggest the presence in the α1(III) chain of a single platelet-reactive site accounting for the total activity of the type III collagen molecule. This contrasts with our earlier study of type I collagen [5] demonstrating the presence of a wide distribution of sites of relatively moderate reactivity and suggests that there may be more structural specificity associated with collagen platelet-reactive sites than we first supposed.

The peptide α1(III)CB4 occurs in exactly the same location in the α1(III) chain (residues 403–551) as the peptide α1(I)CB3 in the α1(I) chain (Fig. 1). The two peptides exhibit a high degree of structural homology [19]. However, α1(I)CB3 is largely unreactive towards platelets in contrast to the high reactivity of α1(III)CB4. Our results using specific amino-acid modification reagents indicate the importance of lysyl residues in the activity of α1(III)CB4. Of the total of eight lysyl residues in this peptide, that at position 486 is the only basic residue present that is absent from α1(I)CB3. It occurs in the sequence GPK(486)GE. We have previously discussed the proposal that a platelet-reactive site may comprise two basic residues situated relatively close to each other and in adjacent α-chains, with a spatial relationship to each other defined by the triple-helical conformation of the collagen molecule. Another lysyl residue occurs in α1(III)CB4 at position 479, close to lysine 486, and in a similar sequence: GK(479)PGE (although the prolyl residue occurs as hydroxyproline). We believe these two residues (one from one chain, one from another) may together constitute the platelet-reactive site in α1(III)CB4 and in collagen type III itself. This particular spacing of

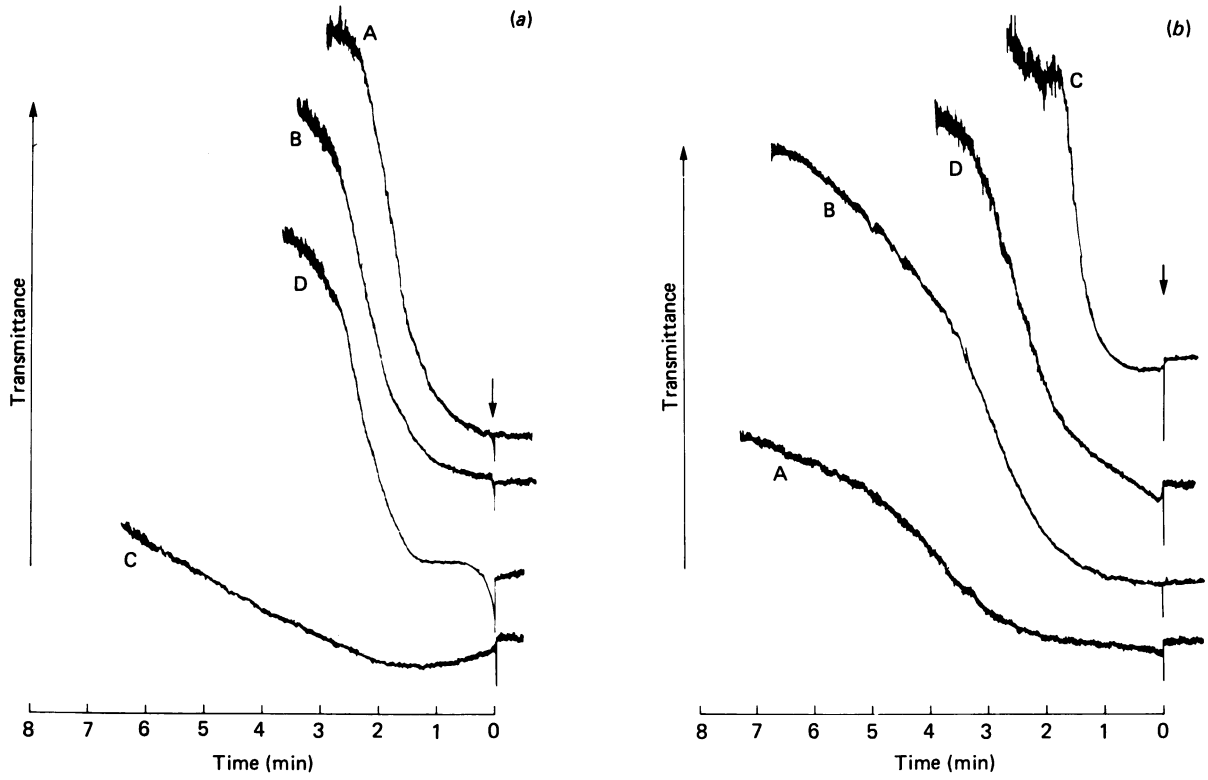


**Fig. 3. Platelet reactivity of the peptides (*a*) α1(III)CB4 and (*b*) α1(I)CB7 after modification of lysyl or arginyl residues**

Peptides were first renatured and polymerized prior to reaction of lysyl residues with trinitrobenzenesulphonic acid and of arginyl residues with phenylglyoxal as described in the Materials and methods section. Following treatment, platelet aggregation was measured at 25 °C, as detailed in the legend to Fig. 1, at the concentrations (μg/ml) indicated below. The arrow indicates the point of addition of sample. Trace A, Phenylglyoxal-treated: (*a*) 5 (0.3), (*b*) 40 (10); B, Control to A: (*a*) 5 (0.5), (*b*) 40 (5); C, Trinitrophenylated: (*a*) 40 (40), (*b*) 40 (5); D, Control to C: (*a*) 5 (0.5), (*b*) 40 (5). Numbers in parentheses refer to the minimum concentration required for activity.

two lysyl residues, i.e. at positions 2 and 9 in the $[GXY]_n$ collagen sequence [GK(479)YGXYGXK(486)], does not occur elsewhere in the α1(III) chain nor at all in either the α1(I) or α2(I) chains of collagen type I [20; P. P. Fietzek & K. Kuhn, unpublished work]. Although we believe that the prolyl (hydroxyprolyl) residues may play a critical conformational role in the sequence, we are uncertain of the extent to which the presence of glutamic acid residues is essential for activity. Support for the importance of the two lysyl residues in question in the platelet reactivity of collagen type III comes from the investigations of Fauvel *et al.* [21] who observed that platelets, preincubated with the peptide α1(III)CB4 under conditions designed to prevent platelet aggregation, were retained on a Sepharose 2B column. Presumably the peptide bound to the column and the platelets then bound to the immobilized peptide. Cleavage of the peptide by various means led to the conclusion that the site associated with adhesion was located between residues 478 and 486. The isolated sequence was found able to inhibit collagen-(type III)-induced platelet aggregation [22], but yet did not prevent the adhesion of platelets to collagen [23]. We ourselves, it should be noted, as we report here, have not detected so far inhibition of collagen-induced platelet aggregation (and so it follows, adhesion to collagen) by collagen-derived peptides lacking tertiary and quaternary structure.

The peptide α1(I)CB7 is the most active of the type-I-derived peptides but the equivalent type-III-derived peptide, α1(III)CB5, is without activity. Our results indicate the importance of arginyl rather than lysyl residues in the activity of α1(I)CB7. The arginyl residue at position 732, absent in α1(III)CB5, occurs in the sequence GPR(732)GE (identical to that of lysine 486 in α1(III)CB4). A second arginyl residue nearby, also absent in α1(III)CB5, occurs at position 740 within the sequence GR(740)PGE (the prolyl residue occurring as hydroxyproline) which is identical to that of lysyl residue 479 in α1(III)CB4. It is tempting, then, to regard these two arginyl residues as the reactive site in α1(I)CB7. The lower reactivity of α1(I)CB7 relative to α1(III)CB4 can be attributed to the presence of arginine rather than lysine and to the rather different spacing of the two basic residues at positions 3 and 11 in the sequence GPR-(732)G(EY)G(XY)GR(740)PG(EY) compared to their location at positions 2 and 9 in the pertinent sequence in α1(III)CB4.

Our results indicated that lysyl residues are crucial for the platelet-aggregatory activity of collagen type-III fibres and in accord with this lysyl residues were found to be essential for the activity of the peptide α1(III)CB4. However, collagen lysyl residues were also necessary for the activity of type I fibres, although aggregation by the peptide α1(I)CB7 was dependent upon the presence of arginyl residues. This suggests that collagen type I

possesses a lysyl-dependent site of relatively high reactivity [comparable to that in α1(III)CB4] that we have yet to locate. Conceivably such a site is present in the α2-chain for we detected no such site in peptides α1(I)CB3, 6, 7 and 8, representing in total 90% of the α1(I) chain [5]. Furthermore, we have shown that the α2(I) chain is the most reactive chain in type I collagen. Thus polymers of the molecule $[\alpha 2(I)]_3$ exhibit a much higher platelet-aggregatory activity than those of $[\alpha 1(I)]_3$ [4]. However, the α2-chain does not contain a GK(2)P...GPK(9) or GPK(3)...GK(11)P type of sequence. The most plausible alternative may be the sequence of residues 406–419: GPK(3).....GK(11)A(GE), but this involves a substitution of a prolyl residue by alanine. Studies with synthetic peptides should ultimately resolve these questions. In collagen type I, anyway, the reactive site may very well comprise a combination of lysyl residues from different α-chains, one from an α1(I)-chain, the second from an adjacent α2(I)-chain. These chains could even be located in different molecules. This would suggest, however, that precise alignment of molecules would be crucial for activity and yet the polymer produced by random association of molecules with glutaraldehyde is nearly as active as the native-type fibre in which molecules are assembled in highly-ordered fashion ([13] and present results).

Although the sequence RGD has been shown to be important in the recognition of platelets by fibronectin, von Willebrand factor and fibrinogen [24,25], and as a general cell-recognition site in fibronectin, vitronectin [26] and collagen [27], our results here, as previously [5], imply that it is not involved in the recognition of platelets by collagen, since it is absent in the peptide α1(III)CB4.

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