Structure of Antigenic Determinants in the *N*-Terminal Region of Dermatosparactic Sheep Procollagen Type I

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About half of the rabbit antisera raised against type-I procollagen, $\alpha 1(I)$ chain or non-reduced procollagen peptides reacted in a radioimmunoassay with the reduced form of peptide Col 1, which comprises the whole non-collagenous region at the *N*-terminus of procollagen. Proteolytic fragments prepared from reduced peptide Col 1 were still effective inhibitors of the antibodies and allowed the localization of two antigenic determinants. The antigenically active regions have the sequences <Glu-Glu-Glu-Gly-Gln-Gln-Glu and Gly-Asp-Thr-Gly-Pro-Arg, and are located at the *N*- and *C*-termini of the peptide respectively. Antibodies raised against reduced peptide Col 1 bind to a determinant localized in a different region of the peptide.

The non-collagenous *N*-terminal segment of type-I procollagen has been previously characterized as a strong antigen (Timpl *et al.*, 1973; Kohn *et al.*, 1974; von der Mark *et al.*, 1973; Rohde *et al.*, 1976a,b). This segment can be isolated from a collagenase digest of procollagen in form of the peptide Col 1, which has a molecular weight of about 10000 (Furthmayr *et al.*, 1972; Becker *et al.*, 1976). Physical studies (Engel *et al.*, 1977) indicated a rigid elongated structure of the peptide that is stabilized by five intrachain disulphide bridges. Two types of antigenic determinants are found in peptide Col 1 (Rohde *et al.*, 1976b). Most antibodies react with conformational determinants that can be destroyed by reducing the disulphide bridges. A minor fraction of the antibodies, however, reacts with determinants shared by the native and reduced forms of the peptide. This observation was consistent with physicochemical data (Engel *et al.*, 1977) indicating that short *N*- and *C*-terminal sequences of peptide Col 1 are not involved in the rigid structure of the molecule. Since the amino acid sequence of peptide Col 1 has been established (Rohde *et al.*, 1979), we now attempted to localize the sequence-dependent antigenic determinants of peptide Col 1 to distinct regions of the peptide.

Materials and Methods

Fragments of procollagen peptide

The procollagen peptide Col 1 either in its native disulphide-bonded or in its reduced and *S*-carboxymethylated forms was isolated from dermatosparactic sheep skin as described previously (Becker

et al., 1976). Tryptic peptides were prepared from reduced peptide Col 1 and purified by chromatographic methods (Rohde *et al.*, 1979). The order and size of these tryptic peptides are shown in Fig. 1. Limited digestion of tryptic peptide T1 (residues 1–24 of peptide Col 1) with staphylococcal proteinase V8 produced the fragments T1SP1 (residues 8–24) and T1SP2 (residues 1–7), whereas the smaller fragments T1SP2a (residues 4–7) and T1SP2b (residues 1–3) were obtained after a more drastic treatment with the same proteinase. Fragment T1SP1a (residues 12–24) was produced from fragment T1SP1 by a second cleavage with staphylococcal proteinase and purified on Bio-Gel P-4. Amino acid analysis shows that cleavage had occurred at the Glu-Asp bond (position 11–12) in fragment T1SP1. Fragments T1a (residues 1–18) and T1b (residues 19–24) were produced from an *S*-aminoethylated variant of peptide T1 by a second trypsin treatment (Rohde, 1977; Rohde *et al.*, 1979). Fragment C1 was prepared from a chymopapain digest (Worthington, Freehold, NJ, U.S.A.; enzyme/substrate ratio 1:30; 24 h at 37°C) of peptide T1 and was purified by chromatography on a Bio-Gel P-4 column equilibrated in 0.2M-ammonium bicarbonate, pH 8.5. Before use the enzyme was activated as described by Ebata & Yasunobu (1962). The position of fragment C1 (residues 1–16) in peptide T1 was established by amino acid analysis.

Antisera

The rabbit antisera to dermatosparactic type-I procollagen, $\alpha 1(I)$ chain and peptide Col 1 were those used in a previous study (Rohde *et al.*, 1976b).

Fig. 1. Order of the tryptic peptides in procollagen peptide Col 1 and of further fragments obtained from peptide T1. The fragments were obtained by cleavage with trypsin (T), staphylococcal proteinase (SP) or chymopapain (C). Numbers in parentheses refer to the numbers of amino acid residues in the peptides.

In addition, we used a single antiserum against reduced and *S*-carboxymethylated peptide Col 1.

Radioimmunoassay

Appropriate dilutions of the antisera were reacted with 1 or 2 ng of ^{125}I -labelled reduced and *S*-carboxymethylated peptide Col 1 (Rohde *et al.*, 1976a). In the inhibition assay fixed amounts of antibody were preincubated with non-labelled antigens before the addition of the labelled antigen. Immune complexes were finally separated from free antigen by precipitation with goat antiserum to rabbit immunoglobulin G. Results of the inhibition assays were expressed in percentage displacement of labelled antigen from antibody as described by Lotter & Timpl (1975).

Results

Four out of nine different antisera produced against various non-reduced procollagen antigens showed a distinct binding to labelled reduced peptide Col 1. The reaction could be equally well inhibited by native and reduced peptide Col 1, indicating that the antigenic determinants involved are shared by both forms of the peptide. A tryptic digest prepared from reduced peptide Col 1 also had strong inhibitory activity (Rohde *et al.*, 1976b).

In the present study we examined by inhibition assays whether antigenic activity could be localized on individual tryptic peptides of peptide Col 1 (Fig. 2). The reaction of a single antiserum to $\alpha 1(\text{I})$ chain (no. 709) could be completely inhibited by the *N*-terminal peptide T1, and the inhibition curve found with T1 was superimposable on that produced

by the whole tryptic digest (Fig. 2a). Other tryptic peptides either were inactive (T2, T6) or showed inhibition (T3-4, T5) only when used at 300-fold higher concentration compared with peptide T1. Presumably the inhibiting activity of peptides T3-4 and T5 is due to a small contamination with peptide T1. Studies with three other antisera (two against peptide Col 1, nos. 667 and 670, one against procollagen, no. 529) showed distinct inhibiting activity for three tryptic peptides, T1, T5 and T6 (Fig. 2b). Since inhibition with each individual peptide did not exceed 30–40% of the total reaction, it is likely that different antibody fractions react with the different peptides. In support of this interpretation we found that an equimolar mixture of peptides T1, T5 and T6 was a better inhibitor than each individual peptide alone, even though the inhibition curve shown by the mixture did not completely approach the curve produced by the whole tryptic digest of peptide Col 1.

Further studies were carried out with the antiserum showing the exclusive reaction with peptide T1 (Fig. 2a) in order to localize antigenic activity on fragments prepared by proteolysis of peptide T1 (see Fig. 1). In the inhibition assay (Fig. 3) peptide T1 (residues 1–24) and its fragments (residues 1–7, 1–16 and 1–18) showed identical activity. Fragments from the central or C-terminal region of peptide T1 (i.e. residues 8–24 and 19–24) were inactive. Cleavage of the active fragment (residues 1–7) to the smaller fragments (residues 1–3 and 4–7) destroyed antigenicity. We have also examined whether fragments with a length of three to eight amino acid residues prepared from peptide T5 by staphylococcal proteinase (Rohde *et al.*, 1979) were antigenically active in an assay system similar to that shown in Fig. 2(b).

Fig. 2. Localization by inhibition radioimmunoassay of antigenic activity on tryptic peptides of the procollagen peptide Col 1

Two antisera against (a) non-reduced pα1(I) chain (no. 709) and against (b) non-reduced peptide Col 1 (no. 670) were reacted against 125 I-labelled, reduced and alkylated peptide Col 1. Inhibitors of the reactions were a tryptic digest of peptide Col 1 (▼), a mixture of the peptides T1, T5 and T6 (■) and the individual tryptic peptides T1 (○), T2 (□), T3-4 (△), T5 (●) and T6 (▲). Two other antisera (nos. 529 and 667) showed a pattern similar to that of (b).

Fig. 3. Localization of an antigenic determinant on peptide T1 by an antiserum to non-reduced pα1 chain

Peptides tested for inhibitory activity were peptide T1 [residues 1-24 (▼)] and fragments of peptide T1 [residues 1-18 (○), 19-24 (△), 1-7 (●), 8-24 (▲), 1-16 (□), 1-3 (▽) and 4-7 (■)].

Fig. 4. Localization of an antigenic determinant on peptide T1 by an antiserum (no. 713) to reduced and alkylated peptide Col 1

Peptides tested for inhibitory activity were peptides T1 [residues 1-24 (▼)] and fragments of peptide T1 [residues 1-18 (○), 19-24 (△), 1-7 (●), 8-24 (▲), 1-16 (□) and 12-24 (▽)].

None of these fragments showed significant inhibition.

An antiserum against reduced peptide Col 1 (no. 713) also showed distinct binding of labelled reduced peptide Col 1. The specificity of this antiserum is, however, different from those examined above

(Figs. 2 and 3), since the reaction could be inhibited by reduced peptide Col 1 but not by native peptide Col 1 (Rohde *et al.*, 1976b). On examination of

tryptic peptides as inhibitors, only peptide T1 was found to be active. In fact, the inhibition curves produced by the various peptides closely resembled those shown for another antiserum in Fig. 2(a). Inhibition studies with proteolytic fragments of peptide T1 (Fig. 4) demonstrated distinct activity on peptide fragments representing residues 1–16, 1–18 and 8–24. Only 2–3-fold higher concentrations of these fragments were required to approach the inhibiting activity of peptide T1. Compared with peptide T1, a 100-fold lower activity was found for the fragment representing residues 12–24, whereas the *N*-terminal and *C*-terminal peptides (residues 1–7 and 19–24 respectively) were inactive.

Discussion

Structural analysis of peptide Col 1 (Rohde *et al.*, 1979; Hörlein, 1978) has demonstrated that the peptide is composed of two different sequence domains. The domain residues 1–86 comprises a non-collagenous segment showing an irregular distribution of glycine, whereas peptide T6 and the last six residues of peptide T5 (residues 87–98) have the collagen-like triplet structure Gly-X-Y. The ten cysteine residues of peptide Col 1 are found entirely in the non-collagenous domain and are distributed between position 18 in peptide T1 and position 73 in peptide T5. The sequence-dependent antigenic determinants that are shared by the native and the reduced peptide Col 1 were found in the present study to be localized to the terminal regions of the peptide. These data resemble observations with other proteins such as myoglobin (Crumpton *et al.*, 1970), unfolded lysozyme and ferredoxin (Young & Leung, 1970; Kelly & Levy, 1971), fibrinogen (Tanswell *et al.*, 1977, 1978) and collagen (Timpl, 1976), where sequence-dependent antigenic determinants have also been identified in terminal positions of the proteins.

Two of the antigenic determinants in native peptide Col 1 were found to be localized to a heptapeptide (T1SP2, residues 1–7) from the *N*-terminus

and to a hexapeptide (T6, residues 93–98) from the *C*-terminus of the molecule. The amino acid sequences of these peptides are shown in Fig. 5. The small size of the peptides suggests that they comprise a single antigenic determinant. Fragments of comparable size have been found in other proteins to be antigenically active (Crumpton, 1974; Timpl, 1976). Cleavage of the Glu-Gly bond in the *N*-terminal heptapeptide abolishes antigenicity, and this in turn suggests that the determinant spans over the entire region of the peptide. The *C*-terminal hexapeptide is derived from the precursor-specific collagenous region of procollagen, which is assumed to exist in a triple-helical conformation in the native protein (Engel *et al.*, 1977). This antigenic determinant apparently becomes exposed on release of peptide Col 1 from procollagen or by denaturation of procollagen that may occur after injecting the protein into the rabbit. A further determinant was found on peptide T5, which is located close to the *C*-terminus of peptide Col 1. Here staphylococcal proteinase produced a number of small fragments (Rohde *et al.*, 1979) that were inactive in the inhibition assay and thus did not allow us to localize the antigenic determinant precisely within the sequence.

In the studies with the antiserum to reduced peptide Col 1 no fragments with less than ten amino acid residues could be identified that still possessed distinct antigenic activity. However, results obtained with appropriate overlapping fragments indicate that the corresponding antigenic structure is localized in residues 8–16 of the sequence of peptide Col 1 (Figs. 4 and 5). The observation that native peptide Col 1 is an ineffective inhibitor of these antibodies (Rohde *et al.*, 1976b) indicates that this region of the molecule contributes to its rigid structure, whereas the adjacent sequence residues 1–7 has a random-coil structure. Since the sequence residues 8–16 does not contain cysteine, the unique antigenic specificity of reduced and carboxymethylated peptide Col 1 cannot be attributed to the chemical modification of the protein.

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- (1–7) <Glu-Glu-Glu-Gly-Gln-Gln-Glu-
 (8–16) -Gly-Gln-Glu-Glu-Asp-Ile-Pro-Pro-Val-
 (93–98) -Gly-Asp-Thr-Gly-Pro-Arg-

Fig. 5. Amino acid sequence of the antigenically active regions in the procollagen peptide Col 1. Numbers in parentheses refer to the positions (residue numbers) within the sequence of peptide Col 1. Region residue numbers 1–7 refer to the data shown in Fig. 3, region residue numbers 8–16 to Fig. 4 and region residue numbers 93–98 to the partially active peptide T6 in Fig. 2(b). The sequence data are taken from Rohde *et al.* (1979).

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