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An Ehrlich chromogen in collagen cross-links

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A well-characterized three-chain peptide [$(Col1)_2 \times T9$] from human type III collagen was a rich source of Ehrlich chromogen. The corresponding two-chain peptide [$(Col1)_2$] was not, implying that the Ehrlich chromogen is a trifunctional cross-link. $(Col1)_2 \times T9$ also contained pyridinoline, which is not an Ehrlich chromogen. The 7S domain of type IV collagen also contained an Ehrlich chromogen.

Proteolytic digests of connective tissues contained material that reacted rapidly at room temperature with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) in acid, to give a cherry-pink colour (Scott *et al.*, 1981). This material, the Ehrlich chromogen, was excluded from Bio-Gel P-2 and Bio-Gel P-4 gel, and, after considerable enrichment, was associated with polypeptides that were collagenous in origin. A number of Ehrlich-reactive compounds occurring in tissues were excluded on the basis of their physical and chemical properties. It was suggested that the Ehrlich chromogen might be a pyrrole, derived from the amino-ketone cross-links of collagen by a Knorr reaction. The generally labile nature of the chromogen, e.g. towards acid hydrolysis and atmospheric oxidation, as well as its resistance to sodium borohydride reduction, were compatible with this proposal, which is further supported by the observation that the Ehrlich chromogen reacts rapidly in acid with diazotized sulphanilic acid to produce a yellow colour (J. E. Scott, E. W. Hughes & A. Shuttleworth, unpublished work).

In the present paper we report results showing the presence of the Ehrlich chromogen in well-characterized cross-linked peptides from human type III and type IV collagen; these findings strongly support the proposal of a cross-linking function of the Ehrlich chromogen.

Experimental

Materials

Two cross-linked peptides $(Col1)_2$ and $(Col1)_2 \times T9$ from type III collagen were isolated from human leiomyoma tissues as previously described (Henkel *et al.*, 1979). The two-chain peptide $(Col1)_2$ contained the *N*-terminal 21 amino acid residues from each of the two α-chains (i.e. 42 residues in all) of type III collagen, and the three-chain peptide $(Col1)_2 \times T9$ contained an additional nine amino acid residues from the *C*-terminal helical cross-linking site of type III collagen. The 7S domain of type IV collagen was prepared from human placenta (Risteli *et al.*, 1980).

Pyridinoline was a gift from Dr. D. Fujimoto, Hamamatsu School of Medicine, Hamamatsu, Japan.

Methods

The Ehrlich reaction was performed as described previously (Scott *et al.*, 1981). The reaction with diazotized sulphanilic acid in acid solution was performed as described by Brodersen (1966).

Excitation and emission fluorescence spectra were obtained with an Aminco Bowman spectrofluorimeter.

Results

The three-chain peptide $(Col1)_2 \times T9$ gave an immediate marked cherry-red colour with Ehrlich reagent, identical in spectral shape (i.e. $\lambda_{max.}$ 572 nm) with, for example, Pronase digests of bone matrix, periosteum and tendon (Scott *et al.*, 1981). The absorbance in a 1 cm cuvette of a 1% solution of the three-chain peptide at 572 nm in the presence of Ehrlich reagent was 2.0. The three-chain peptide $(Col1)_2 \times T9$ also gave a yellow colour with diazotized sulphanilic acid, and in 0.1 M-HCl the three-chain peptide $(Col1)_2 \times T9$ gave fluorescence excitation and emission spectra very similar to those

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of pyridinoline ($\lambda_{max.}$ 290nm and $\lambda_{max.}$ 400nm respectively). Pyridinoline and the three-chain peptide $(Col1)_2 \times T9$ dissolved in the same solution gave the expected sum of their fluorescence contributions, i.e. neither quenced the other.

The two-chain peptide $(Col1)_2$ gave almost no colour with Ehrlich reagent [i.e. less than 5% of that of the three-chain peptide $(Col1)_2 \times T9$].

The 7S domain of type IV collagen gave an immediate pink colour with Ehrlich reagent, which was spectrally somewhat different from that of the three-chain peptide $(Col1)_2 \times T9$, with two roughly equal peaks, at 530 and 572nm. The $A^{1\%}_{1cm}$ at 572nm was 0.05. The polypeptides from CNBr cleavage of this preparation gave no Ehrlich reaction.

Pyridinoline gave no reaction with Ehrlich reagent, or with diazotized sulphanilic acid.

Discussion

The cross-linking three-chain peptide $(Col1)_2 \times T9$ is the best source of the Ehrlich chromogen yet encountered in polypeptides prepared from connective tissue. The $A^{1\%}_{1cm}$ value at 572nm is about 4 times that of the most enriched preparation from Pronase-digested bone that we have so far achieved after purification and fractionation. On the other hand, the closely related two-chain peptide $(Col1)_2$ contained little Ehrlich chromogen. This difference points to the conclusion that a trifunctional cross-link, present in the three-chain $(Col1)_2 \times T9$, but absent from the two-chain peptide $(Col1)_2$, must be the chromogen, since there is nothing in the nine-amino-acid-residue peptide T9 (Gly-Ala-Met-Gly-Ile-Xaa-Gly-His-Arg) that would be expected to react with Ehrlich reagent, apart from the trifunctional cross-link Xaa. The two-chain peptide $(Col1)_2$ is cross-linked by a reducible aldol-condensation product (Becker *et al.*, 1976), which clearly is not an Ehrlich chromogen.

The molecular weight of the three-chain peptide $(Col1)_2 \times T9$ is close to 5000, and, if the only trifunctional link present were the Ehrlich chromogen, the molecular absorption coefficient would be 1000. If the Ehrlich chromogen were pyrollic, the value would be expected to be somewhat higher (approx. 10000), although the effects of substituents could be very marked. However, the fluorescence spectra suggest strongly that pyridinoline is also present in the three-chain peptide $(Col1)_2 \times T9$. Assuming 1 mol of cross-link per mol of three-chain peptide, about 25% of the cross-link could be pyridinoline, on the basis of spectrofluorimetric assay, with a pyridinoline standard. The u.v. absorption at 326nm, in neutral and alkaline solution, which is characteristic of pyridinoline (Fujimoto *et al.*, 1977), and unlikely to be due to other components of the three-chain peptide $(Col1)_2 \times T9$, also suggested that about 25% of the cross-link could be pyridinoline. Since pyridinoline does not react with Ehrlich reagent or with diazotized sulphanilic acid, it seems that the three-chain peptide $(Col1)_2 \times T9$ contains at least two compounds that might act as cross-links. This is not surprising in view of the very reactive nature of the difunctional keto-amine cross-links, which could 'mature' in many different ways.

The structure of pyridinoline suggests a trifunctional capacity. It remains to be seen whether the Ehrlich chromogen is an exact analogue.

The demonstration of Ehrlich chromogen(s) in type IV collagen is interesting, particularly since it is present in the 7S domain, in which four triple-helical molecules are firmly linked together (Kuhn *et al.*, 1981). The shape of the spectrum of the Ehrlich product suggests that the structure of the chromogen(s) may not be the same as those from the interstitial collagens, underlining the comments, above, on the possible multiplicity of matured forms of cross-links. CNBr apparently converts type IV chromogen into an unreactive form, as might be expected of a pyrrole. If this is found to be true of Ehrlich chromogens from other collagens, cleavage by CNBr will not be a suitable degradation method to use in the search for these cross-links.

References

Becker, U., Fietzek, P. P., Nowack, H. & Timpl, R. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357**, 1409–1415

Brodersen, R. (1966) *Scand. J. Clin. Lab. Invest.* **18**, 361–379

Fujimoto, D., Akiba, K. & Nakamura, N. (1977) *Biochem. Biophys. Res. Commun.* **76**, 1124–1129

Henkel, W., Rauterberg, J. & Glanville, R. W. (1979) *Eur. J. Biochem.* **96**, 249–256

Kuhn, K., Wiedermann, H., Timpl, R., Risteli, J., Dieringo, H., Voss, T. & Glanville, R. W. (1981) *FEBS Lett.* **125**, 123–128

Risteli, J., Bachinger, H. P., Engel, J., Furthmeyr, H. & Timpl, R. (1980) *Eur. J. Biochem.* **108**, 239–250

Scott, J. E., Hughes, E. W. & Shuttleworth, A. (1981) *Biosci. Rep.* **1**, 611–618