THE NATURE OF A HYDROXYLAMINE-SENSITIVE BOND IN COLLAGEN

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SUMMARY

A cyanogen bromide peptide (α1-CB8) from the α1 chain of rat collagen was cleaved with hydroxylamine. Isolation of the relevant tryptic peptides from a resistant fraction of α1-CB8 and from the two hydroxylamine-produced fragments permitted the characterization of the susceptible bond. The data indicate that degradation of α1-CB8 results from cleavage of a cyclic imide, the derivative of a normal peptide bond which forms by condensation of an aspartyl (or asparaginyl) side chain with the subsequent amide nitrogen in the polypeptide chain. Such cyclic imides may represent the bonds presumed to link subunits within collagen chains.

The subunit model of collagen structure provides for the existence in each α chain (MW 95,000) of five or six lower molecular weight polypeptides linked by "ester-like" or special imide bonds which are sensitive to hydroxylamine and hydrazine (Gallop et all, 1967). Several lines of evidence indicate that both α- and β-carboxyl groups of aspartic acid are involved in the proposed subunit attachment sites (Blumenfeld and Gallop, 1962).

Although the accumulated data supporting the existence of subunits within a collagen chain are considerable, studies using nucleophilic reagents have been limited to the intact protein. The resulting peptide mixtures are too complex to resolve and consequently the available evidence regarding the nature of the susceptible bonds is largely indirect. In order to characterize a specific nucleophile-sensitive bond, α1-CB8 a CNBr-produced fragment from the α1 chain of rat collagen (Butler et al., 1967), was treated with hydroxylamine. The findings to be described do not support the concept that ester or other non-peptide bonds are present within collagen chains.

METHODS AND MATERIALS

The α1 chains of rat skin and tail tendon collagen were prepared by

chromatography on CM cellulose (Piez et al., 1963). CNBr-produced fragments were separated and purified on CM cellulose at pH 3.6 and 4.8 (Butler et al., 1967). Hydroxylamine cleavage was performed at 35° C for 90 minutes at pH 10.5, (Blumenfeld et al., 1965). Hydroxylamine-produced fragments were separated by chromatography on Agarose 1.5 M (Bio-Rad) equilibrated with 1 M $CaCl_2$, 0.05 M Tris-HCl, pH 7.5. Calibration of the column using collagen peptides of known size permitted calculation of the molecular weight of the cleavage products (Piez, 1968).

Hydroxamate was detected colorimetrically following oxidation to nitrite (Bergmann and Segal, 1956). Hydrazinolysis was performed using an Amberlite CG-50 catalyst (Braun and Schroeder, 1967). The α- and β-hydrazides of aspartic acid were synthesized by hydrazinolysis of α- and β-aspartyl benzyl esters and separated by high voltage electrophoresis in 0.5 M acetic acid, pH 2.42.

Tryptic digests of α1-CB8 and of its hydroxylamine-produced fragments were chromatographed on cation and anion exchange resins using volatile buffers. Ninhydrin analyses of hydrolyzed aliquots of the effluent stream were performed with an automatic apparatus. Sequence analysis was performed by sequential dansylation and Edman degradation (Bornstein, 1969). Dansyl-amino acids were identified by thin layer chromatography.

RESULTS

Degradation of α1-CB8 occurred consistently in 1 M hydroxylamine, pH 10.5. Cleavage was less complete in 1 M hydrazine. Dilute alkali alone and 1 M piperidine at pH 11.0 were ineffective.

Analysis of incubation mixtures of α1-CB8 with hydroxylamine revealed three peaks which were separable by agarose chromatography (Figure 1a) and acrylamide gel electrophoresis. The largest of these was identical with α1-CB8 in its position of elution and amino acid composition. The sum of the amino acid compositions and molecular weights of the other two fragments, HA2

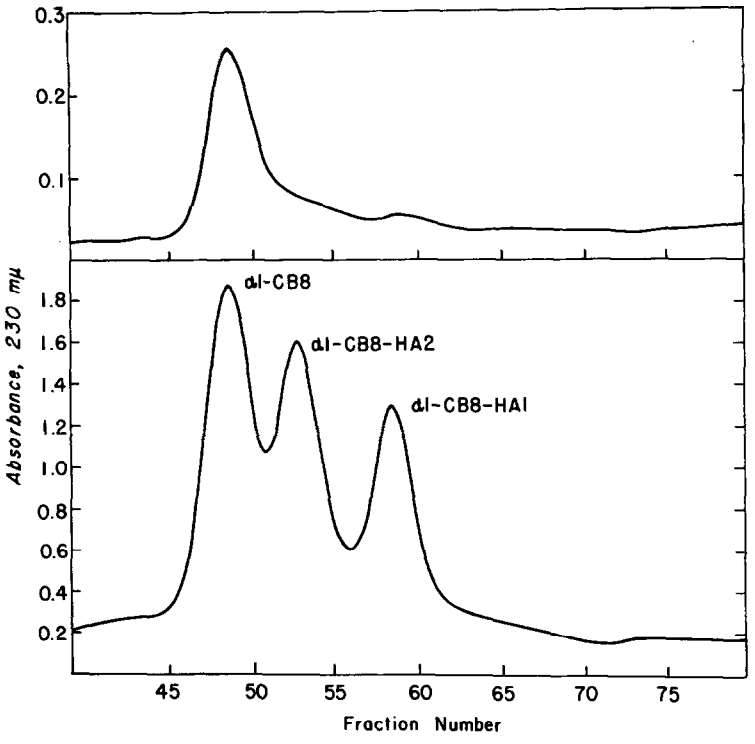

Figure 1. (a) below, agarose elution pattern of fragments obtained by cleavage of α1-CB8 with hydroxylamine.
(b) above, agarose elution pattern of α1-CB8 which was resistant to hydroxylamine cleavage, retreated with hydroxylamine.

and HA1, accounted within experimental error for the parent peptide (Table I). HA2 contained 1 residue of homoserine/peptide whereas most of the hydroxamate was present in HA1. These findings indicate that cleavage occurred at a point approximately one third of the distance from the NH_2-terminal end of α1-CB8. The presence of hydroxamate in the uncleaved peptide and in HA2 can be attributed to hydroxylaminolysis at the COOH-terminal homoseryl residue which exists in part as the cyclic ester or lactone.

When the α1-CB8 fraction which was not cleaved was isolated and retreated with hydroxylamine, little additional cleavage occurred (Figure 1b) indicating that the persistence of α1-CB8 is not due to incomplete reaction with the nucleophile. Pretreatment of α1-CB8 at 35° for 1 hour in 0.2 M K_2CO_3 buffer, pH 10.5, resulted in more complete stabilization of the fragment to

Table I

Molecular weight and hydroxamate content of the reaction products of α1-CB8 treated with hydroxylamine.

Peptide	Molecular Wt.	μmoles hydroxamate / μmole peptide
α1-CB8	24,000	0.6
α1-CB8-HA1	7,500	1.3
α1-CB8-HA2	13,500	0.4

subsequent hydroxylamine cleavage. The starting material, α1-CB8, is therefore heterogeneous in that the hydroxylamine-sensitive bond exists in at least two forms. The stable fraction, and large chymotryptic peptides derived therefrom, display elution characteristics during chromatography on CM cellulose consistent with the presence of an additional negative charge.

Chromatography of a tryptic digest of HA1 on Bio-Gel P2 separated hydroxamate-containing fractions from the bulk of the tryptic peptides (Figure 2). Amino acid analyses suggested the presence in fractions 23-29 of two peptides, a tripeptide composed of glycine, proline and arginine and a second tripeptide composed of glycine, alanine and aspartic acid. The molar ratio of hydroxamate to the aspartic acid-containing tripeptide was 0.93.

The tripeptides in fractions 23-29 were separated by high voltage electrophoresis at pH 3.6. The most cathodally migrating peptide was identified as the arginyl peptide and lacked hydroxamate. The remaining two peptides both contained glycine, alanine and aspartic acid in equivalent amounts and both contained hydroxamate. The stoichiometry of the hydroxamate was difficult to evaluate due to losses which occurred during elution of the peptides from paper (see Gallop *et al.*, 1960). However, the separation at pH 3.6 of two forms of a peptide containing aspartic acid and hydroxamate was consistent with the presence of both α- and β-hydroxamates of peptidyl aspartic acid. Comparable electrophoretic separation of the α- and β-benzyl esters of aspartic acid was observed.

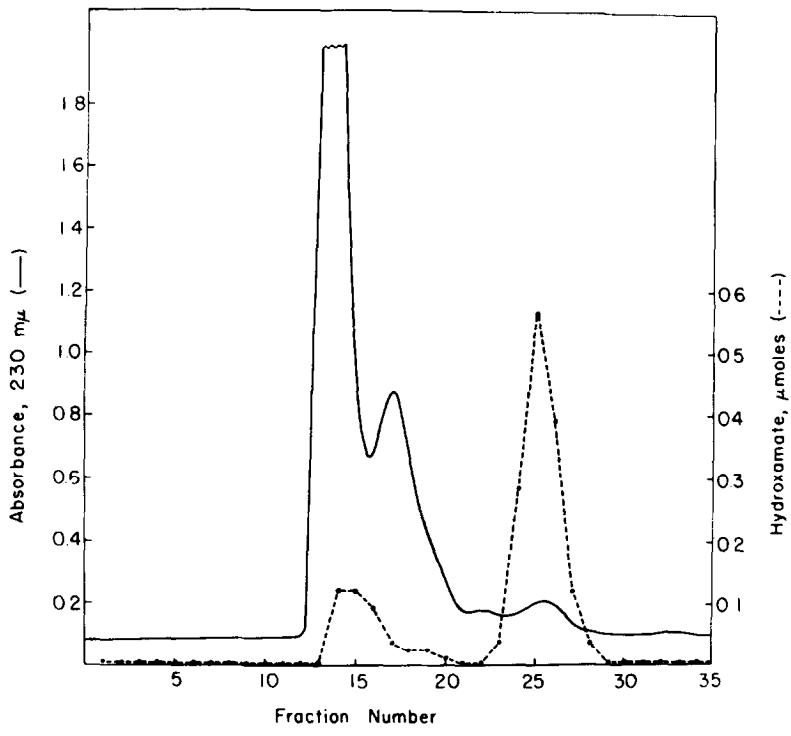

Figure 2. Bio-Gel P-2 elution pattern of a tryptic digest of α1-CB8-HA1.

Hydrazinolysis of fraction 23-29 and separation of the products by electrophoresis at pH 2.42 revealed the presence of both α- and β-aspartyl hydrazides. However, this finding does not demonstrate the prior existence of the corresponding hydroxamates since the hydrazides may conceivably be interconvertible through the dihydrazide (Narita and Ohta, 1959). Sequence analysis of the aspartyl peptides indicated the primary structure to be Gly-Ala-Asp.

The tryptic peptides of the resistant fraction of α1-CB8, and of HA1 and HA2, were resolved by a combination of ion exchange chromatography and paper electrophoresis. An NH_2-terminal glycine octadecapeptide with the amino acid composition $Asp_1, Hyp_3, Gly_6, Ala_5, Ile_1, Phe_1, Arg_1$ was isolated in digests of the hydroxylamine-resistant peptide, but was missing from digests of HA1 and HA2. However, the digest of HA2 contained an NH_2-terminal glycine pentadecapeptide, $Hyp_3, Gly_5, Ala_4, Ile_1, Phe_1, Arg_1$, and that of HA1 contained the tripeptide Gly-Ala-Asp. Since the isoleucyl residue is unique in α1-CB8, any remaining am-

biguity in identification of the hydroxylamine-susceptible region due to the repetitive sequence of collagen can be excluded. These findings clearly indicate that the hydroxylamine-sensitive link is located in the isoleucine-containing octadecapeptide and that an aspartyl-glycyl bond is cleaved in the parent peptide.

DISCUSSION

The following findings indicate that the bond cleaved by hydroxylamine in α1-CB8 represents a cyclic imide formed by the condensation of an aspartyl (or asparaginyl) side chain with the amide nitrogen of the subsequent (glycyl) residue in the polypeptide chain (Figure 3). (a) Treatment with dilute alkali stabilizes the chain to nucleophilic attack and introduces an additional negative charge presumably by saponification of the imide with the liberation of an aspartyl side chain. (b) Two electrophoretic forms of the tripeptide Gly-Ala-Asp, consistent with the existence of α- and β-hydroxamates, were identified. (c) The appearance of a new glycine NH_2-terminal in HA2, coupled with the partial characterization of the tryptic peptides adjacent to the cleaved bond, indicate that a peptide bond or a derivative thereof is cleaved.

There is ample precedent in peptide chemistry for cyclization of aspartyl and asparaginyl peptides with subsequent opening of the ring to form α- and β-aspartyl bonds (John and Young, 1954). The presence of β-aspartylglycine in enzymatic digests of collagen and in the urine of patients fed gelatin

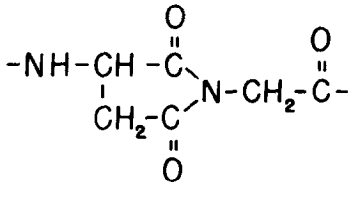

Figure 3. Proposed structure of the hydroxylamine-sensitive bond in α1-CB8. Cleavage of the cyclic imide yields a mixture of α and β aspartyl hydroxamates and a new NH_2-terminal glycine.

(Haley et al., 1966; Pisano et al., 1960) may be a consequence of such cyclization. Base-catalyzed hydrolysis of the cyclic imide of peptidyl aspartic acid occurs readily (Battersby and Robinson, 1955; Swallow and Abraham, 1958). The mechanism by which nucleophilic attack of a substituted cyclic imide with hydroxylamine can lead to chain cleavage and liberation of a free amino group is not completely understood. However, a similar reaction involving the removal of a phthalimido blocking group by hydrazinolysis is well known in peptide synthesis (Schröder and Lubke, 1965). Gallop et al. (1960) reported that treatment of polyanhydroaspartic acid with hydroxylamine, under the conditions used in this work, resulted in extensive degradation of the polymer indicating that hydroxylamine can cleave a chain formed by cyclic imide bonds.

Whether cyclic imides in collagen result from a physiologically significant process or are an artifact of preparation remains to be determined. In any event, the possibility that cyclic imides represent the hydroxylamine-sensitive linkages in collagen explains many of the chemical findings of Gallop and his coworkers and is still compatible with the likelihood that the polypeptide backbone of the protein contains only peptide bonds.

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REFERENCES

Battersby, A. R., and Robinson, J. C., J. Chem. Soc., p. 259 (1955).

Bergmann, F., and Segal, R., Biochem. J. 62, 542 (1956).

Blumenfeld, O. O., and Gallop, P. M., Biochemistry 6, 947 (1967).

Blumenfeld, O. O., Rojkind, M., and Gallop, P. M., Biochemistry 4, 1780 (1965).

Bornstein, P., Biochemistry 8, 63 (1969).

Braun, V., and Schroeder, W. A., Arch. Biochem. Biophys. 118, 241 (1967).

Butler, W. T., Piez, K. A., and Bornstein, P., Biochemistry 6, 3771 (1967).

Gallop, P. M., Blumenfeld, O. O., and Seifter, S., in Treatise on Collagen, Vol. I, edited by G. N. Ramachandran, Academic Press, Inc., New York, p. 339 (1967).

Gallop, P. M., Seifter, S., Lukin, M., and Meilman, E., J. Biol. Chem. 235, 2618 (1960).

Haley, E. E., Corcoran, B. J., Dorer, F. E., and Buchanan, D. L., Biochemistry 5, 3229 (1966).

John, W. D., and Young, G. T., J. Chem. Soc., p. 2870 (1954).

Narita, K., and Ohta, Y., Bull. Chem. Soc. Japan 32, 1023 (1959).

Piez, K. A., Anal. Biochem. 26, 305 (1968).

Piez, K. A., Eigner, E. A., and Lewis, M. S., Biochemistry 2, 58 (1963).

Pisano, J. J., Prado, E., and Freedman, J., Arch. Biochem. Biophys. 117, 394 (1966).

Schröder, E., and Lubke, K. in The Peptides, Vol. I, Academic Press, Inc., New York, p. 9 (1965).

Swallow, D. L., and Abraham, E. P., Biochem. J. 70, 364 (1958).