Vol. 89, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 12, 1979 Pages 202-208

ISOLATION AND CHARACTERIZATION OF A PEPTIDE CONTAINING THE SITE OF CLEAVAGE OF THE CHICK SKIN COLLAGEN α1[I] CHAIN BY ANIMAL COLLAGENASES*

John H. Highberger, Clare Corbett and Jerome Gross

Developmental Biology Laboratory, Medical Services
Massachusetts General Hospital and Department of Medicine
Harvard Medical School, Boston, Massachusetts 02114

Received June 4,1979

SUMMARY: A 36-residue peptide containing the bond cleaved by animal collagenases was isolated from a digest of chick skin collagen α1-CB7 by Staphylococcus V8 protease. This cleavage site peptide, in contrast to the 36-residue α1-CB2, showed no tendency to renature to the triple helical form, as monitored by molecular sieve chromatography and the determination of circular dichroism spectra. These results provide a direct demonstration that the conformation of the α1[I] chain immediately around the collagenase cleavage site in the native molecule must be of a lower degree of helicity than other portions of the chain. This is considered to be an important factor in the collagenase specificity, in providing access to the sensitive bonds, but enzyme binding sites, probably located in the adjacent region(s) of maximum helicity, are also considered necessary to produce the maximum reaction rate.

INTRODUCTION: As the first step in the physiological degradation of collagen, animal collagenases (EC.4.24.7)(1) cleave the native triple helical molecule of molecular weight 285,000, in a highly specific manner at a single locus in each of the three chains at a point about 75% of the chain length from the amino terminus (2). The peptide bonds cleaved (residue 775-776[1]) are one Gly-Ile in each α-1 chain, and one Gly-Leu in α-2 (3,4). Other Gly-Ile and Gly-Leu are not affected.

It is well known that the tightly coiled helical structure of the molecule renders it highly resistant to the action of most proteases, because of the inaccessability of sensitive peptide bonds. Amino acid sequence determinations around the cleavage site in collagen of two different species (6,7) have shown that minimal requirements for the formation of the collagen triple helix are met and that therefore primary structural variation, leading to localized helical disruption at the

*This is publication No.779 of the Robert W.Lovett Memorial Group for the Study of Diseases Causing Deformities. This work was supported by National Institutes of Health grant AM 3564 and a grant from Hoffmann-La Roche Company

[1]Residue numbering begins with the first residue of the helical portion of the chain. The numbers cited in this paper have been corrected for a recently discovered (5) additional triplet in α1-CB7.

0006-291X/79/130202-07$01.00/0

cleavage site, cannot be a factor in this specificity. It has previously been suggested (2,8) that the helical structure in the region of the cleavage site might be of a "loose", or less tightly coiled nature, thus permitting access of the enzyme to the sensitive bonds.

Recently it has become evident that it should be possible to isolate a peptide containing the site of animal collagenase cleavage of the α1[I] chain from a digest of the appropriate segment of the chain (α1-CB7) by the enzyme S.aureus V8 protease (9), whose action can be restricted essentially to the cleavage of glutamyl bonds. Since the glycyl-isoleucyl bond cleaved by animal collagenases is between residues 775 and 776, and the nearest glutamyl residues on each side are at 755 and 791, cleavage at these would result in a 36-residue peptide in which the collagenase site is nearly in the center. Study of the helix-coil transition properties of this peptide, and comparison with those of the CNBr peptide α1-CB2 (10), which is of exactly the same size, would give an opportunity of assessing the probable helical stability of the peptide chain around the cleavage site in the native molecule.

In the present communication we describe the isolation from chick skin collager α1-CB7, and the helix-coil transition characteristics of, this 36-residue peptide which will be referred to herein as the cleavage site (CS) peptide. For comparison the helix-coil transition properties of chick skin collagen α1-CB2 are also described. A similar characterization of rat skin collagen α1-CB2 has previously been reported by Piez and Sherman (10).

MATERIALS AND METHODS: Preparation of Chick Skin Collagen α1-CB2 and α1-CB7

Chick skin collagen was prepared from 3-week old White Leghorn lathyritic chicks, and α1-CB7 and α1-CB2 were isolated from the CNBr digest of this collagen, as described by Kang et al.(11). α1-CB2 was further purified as described by Piez and Sherman (10). An analysis of the peptide as used is given in Table I, and its sequence (12) is shown in Figure 2.

Digestion of α1-CB7 by S. aureus V8 Protease

α1-CB7 was dissolved in 0.05 M NH_4HCO_3, pH 7.80, containing 0.05% Na azide, at a concentration of approximately 1 mg/ml. The solution was heated in a bath at 50° for 20-30 min. After cooling, S.aureus V8 protease (9)(Miles Laboratories) dissolved in the same buffer at a concentration of 1 mg/ml was added in amount sufficient to make an enzyme to substrate ratio of 1:30. The solution was placed

TABLE I

AMINO ACID ANALYSES OF CHICK SKIN COLLAGEN CS PEPTIDE AND α1-(I)CB2

	CS Peptide	Chick Skin Collagen α1-CB2
Hyp	2.49	5.34
Asp	1.12	0.12
Thr	0.85	
Ser	0.77	1.03
Glu	3.82	4.25
Pro	3.33	6.20
Gly	12.12	12.58
Ala	3.44	3.49
Val	1.61	
Ile	1.52	
Leu	0.85	0.93
Phe		1.05
Hyl	0.13	
Lys	1.10	
Arg	1.91	0.98
Hse		0.63*
Total Residues	36	36

Results are given as residues per peptide. A blank indicates less than 0.1 residue found.
*Includes homoserine lactone .

at 37° for approximately 24 hours. At the end of this time another similar aliquot of enzyme was added and the solution was incubated at 37° for an additional 24 hours. It was then frozen and lyophilized.

The enzymatic digest was fractionated on a 2.5 x 110 cm previously calibrated column of Sephadex G-50s in 0.03 M Na acetate, pH. 4.8 at 35°. A typical chromatogram is shown in Figure 1. Eight reasonably well-separated peaks were obtained, of which Peak 7, corresponding to a molecular weight of 3,150, appeared to be the CS peptide. Fractions belonging to this peak were pooled, desalted on Biogel P-2 in 0.1 N acetic acid and rechromatographed on Biogel P-10 in 0.1 N acetic acid. The amino acid analysis of the CS peptide thus purified is given in Table I.

Amino Acid Analyses: Samples for amino acid analysis were hydrolyzed under nitrogen for 24 hours at 110° in glass distilled constant boiling HCl. The analyses were run on a Beckman up-dated 121 analyzer with Autolab System AA Integrater, using the buffer system of Trelstad and Lawley (13). No corrections were made for destruction of amino acids during hydrolysis.

Sequencing the CS Peptide: In order to be certain that the peptide isolated from Peak 7 of the S. aureus protease digest of α1-CB7, as described above, was in fact the CS peptide, it was sequenced, using the methods already described (5). The sequence is shown in Figure 2.

Chromatography of Renatured α1-CB2 and of the CS Peptide: α1-CB2 and the CS peptide were subjected to renaturing conditions by dissolving in 0.015 M potassium acetate, pH 4.8, at a concentration of 5 mg/ml, and maintaining the solution in a bath at 5° for 3 days. The solutions were then chromatographed on a 1.5 x 60 cm column of Biogel P-10 at the same temperature and in the same solvent. The column effluents were monitored by continuous determination of absorbance at 226 nm.

Determination of Circular Dichroism Spectra: Circular dichroism (CD) spectra of the CS peptide and of α1-CB2 were determined using a Cary Model 60 Recording Spectropolarimeter fitted with a Model 6001 CD accessory. The solutions were made

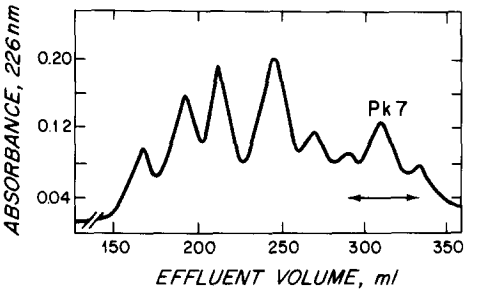


Figure 1. Elution profile of S. aureus V8 protease digestion of 30 mg chick skin collagen α1-CB7 (see text for details) on a 2.5 x 110 cm column of Sephadex G-50s in 0.03 M Na acetate,pH 4.8, at 35°. Flow rate was 21 ml/hr and fractions of 2.8 ml were collected. The horizontal bar indicates how fractions were pooled for isolation of the cleavage site peptide. Tritiated water eluted in this run at 491.5 ml.

Gly-Pro-Ala-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-
Gly-Ala-Hyp-Gly-Pro-Gln-Gly-Phe-Gln-Gly-Pro-Hyp-
Gly-Glu-Hyp-Gly-Glu-Hyp-Gly-Ala-Ser-Gly-Pro-Met

a

Lys-Gly-Ser-Hyp-Gly-Ala-Asp-Gly-Pro-Ile-Gly-Ala-
Hyp-Gly-Thr-Pro-Gly-Pro-Gln-Gly↓Ile-Ala-Gly-Gln-
Arg-Gly-Val-Val-Gly-Leu-Hyp-Gly-Gln-Arg-Gly-Glu

b

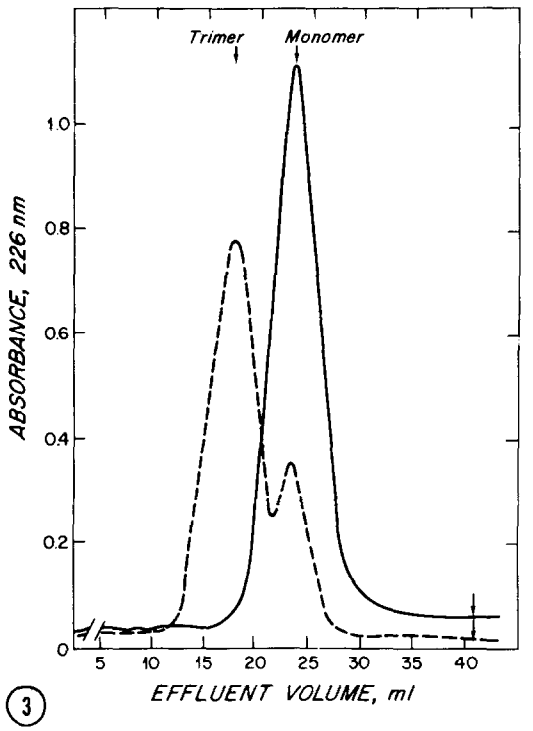
Figure 2. Amino acid sequences of: a, chick skin collagen α1-CB2 (13); b, chick skin collagen CS peptide. Arrow indicates bond cleaved by animal collagenases.

at the concentrations described above and renatured in the same way, except that 0.15 M potassium fluoride adjusted to pH 4.8 with a trace of sulfuric acid replaced the acetate buffer.

After renaturation the solutions were quickly diluted about 30 times with cold 0.15 M potassium fluoride,pH 4.8, in order to bring the readings on scale, and placed in a jacketed cell maintained at 4.5°. After determination of the CD spectrum at this temperature, the temperature was raised to 45°, and the spectrum was redetermined after establishment of equilibrium. The concentrations of the solutions used were determined by amino acid analysis of aliquots of the dilutions measured.

RESULTS AND DISCUSSION:

The chromatograms,obtained when solutions of α1-CB2 and of the CS peptide, kept under renaturing conditions for 3 days, were run on a gel filtration column of P-10 under the same conditions,areshown in Figure 3. It is seen that, as first shown by Piez and Sherman (10) for the rat skin collagen α1-CB2, the bulk of the chick skin collagen α1-CB2 is eluted at a position corresponding to a trimer, due



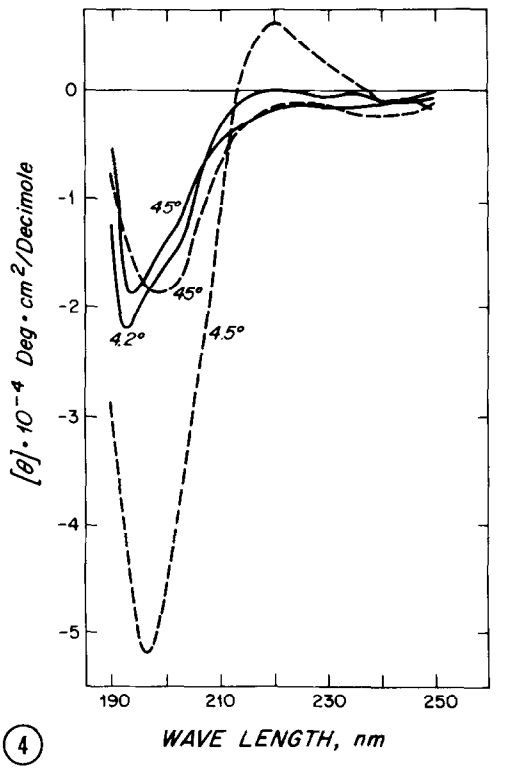


Figure 3. Chromatography of renatured solutions of α1-CB2 and of the CS peptide on a 0.9 x 53 cm column of Biogel P-10, 200-400 mesh, in 0.15 M sodium acetate, pH 4.8, at 5°. Flow rate was 46.0 ml per hr, and fractions of 2.3 ml were collected. Dashed lines, α1-CB2. Solid lines, CS peptide. Details of the prior renaturation are given in the text.

Figure 4. Circular dichroism spectra of α1-CB2 and of the CS peptide, each equilibrated under renaturing (4.2°,4.5°) and denaturing (45°) conditions. Dashed lines, α1-CB2, Solid lines, CS peptide. Details are given in the text.

to the association of 3 monomeric molecules into the triple helical form. Only a small fraction of the total remains in the monomeric form. The CS peptide however elutes completely in the monomeric form, and shows no trace of a peak in the position of the trimer.

This observation was confirmed by the results of the determination of the CD spectra, made as described above. The results of these measurements are shown in Figure 4, plotted as mean residue ellipticities. Whereas renatured α1-CB2 shows a large negative Cotton effect at 197 nm, characteristic of the collagen triple helix, the CS peptide does not. Very little difference is observed between spectra for CS

peptide solutions exposed to renaturing (4.2°) and denaturing (45°) conditions. Both give curves indicative of the random coil form.

Reference to the composition of the CS peptide, as compared to that of α1-CB2, in Table I, and to the sequences of Figure 2, show that the reason for the difference in behavior of the two peptides is very probably their differing content of imino acids. In α1-CB2, whose helix-coil transition behavior is very similar to that of the whole collagen molecule itself, proline and hydroxyproline together amount to 12 residues, one-third of the total, 6 of which are hydroxyproline. In the CS peptide, however, total imino acids amount to only 6, 3 of which are hydroxyproline, and this is not high enough to force the molecule to assume the conformation of the polyproline helix. It is worth noting, also, that the proline at position 16 of the CS peptide is one of only 4 unhydroxylated prolines in the Y position of the triplet, in the whole α1[I] chain.

These results provide a direct demonstration that in the native collagen molecule the conformation of the α1[I] chain immediately around the collagenase cleavage site is of a lower degree of helicity than other portions of the chain. The presence of a unique perturbation in the distribution of bulky residues in the cleavage site region (14) may also contribute to collagenase susceptibility at this locus. We believe that this conformation is an important factor in the collagenase specificity, in providing access to the sensitive bond, but that enzyme binding sites, probably located in the adjacent fully helical region(s), are also necessary for maximum reaction rates. The fact that gelatin (denatured collagen) is cleaved much more slowly than native helical collagen (15) supports this interpretation.

After the completion of this work the paper of Kleinman et al (16) appeared, describing the isolation of the homologous peptide from rat skin collagen α1-CB7. These workers showed that this peptide contains the binding site for the cell attachment protein of serum. It is possible that the conformational factor demonstrated in the present work is also important in this case.

REFERENCES

1. Gross, J. and Lapiere, C.M. (1962), Proc. Natl. Acad. Sci. USA 48:1014-1022.
2. Gross, J. and Nagai, Y. (1965), Proc. Natl. Acad. Sci. USA 54:1197-1204.

3. Nagai, Y., Lapiere, C.M. and Gross, J. (1974), Proc. Int. Congr.Biochem. 6th. 2, 170.
4. Gross, J., Harper,E., Harris, E.D.,Jr., McCroskery,P.A., Highberger, J.H., Corbett, C. and Kang, A.H.(1974), Biochem. Biophys.Res. Commun.61:605-612.
5. Highberger, J.H., Corbett, C., Kang, A.H. and Gross, J. (1978), Biochem. Biophys. Res. Commun. 83:43-49.
6. Fietzek, P.P., Rexrodt, F.W., Hopper, K.E., Kuhn, K. (1973), Eur. J. Biochem. 38:396-400.
7. Highberger, J.H., Corbett, C., Kang, A.H. and Gross, J.(1975), Biochemistry 14:2872-2881.
8. Weiss, J.B.(1976), Int. Rev. Conn. Tissue Res. 7:101-157.
9. Houmard, J. and Drapeau, G.R.(1972), Proc. Natl. Acad. Sci. USA 69:3506-3509.
10. Piez, K.A. and Sherman, M.R.(1970), Biochemistry 9:4129-4133; 4134-4140.
11. Kang, A.H., Piez, K.A. and Gross, J. (1969), Biochemistry 8:1506-1514.
12. Kang, A.H. and Gros, J. (1970), Biochemistry 9:796-804.
13. Trelstad, R.L. and Lawley, K.R. (1976), Anal. Biochem. 70:287-289.
14. Gross, J., Highberger, J.H., Johnson-Wint, B. and Biswas, C. (1979),in Collagenase in Normal and Pathological Connective Tissues, eds, D.E. Woolley and J.M. Evanson, John Wiley and Sons, Ltd, Chichester, England, in press.
15. McCroskery, P.A., Wood, S. Jr., and Harris, E.D., Jr. (1973), Science 182:70-7
16. Kleinman, H.K., McGoodwin, E.B., Martin, G.R., Klebe, R.J., Fietzek, P.P., and Woolley, D.E. (1978), J. Biol. Chem. 253:5642-5646.