RENATURATION AND ORDERING BY ELECTRON MICROSCOPY OF THE CYANOGEN BROMIDE PEPTIDES FROM THE α2 CHAIN OF CHICK SKIN COLLAGEN*

Shogo Igarashi, Andrew H. Kang**, and Jerome Gross

From the Developmental Biology Laboratory, Departments of Medicine, Harvard Medical School, and Massachusetts General Hospital, Boston, Massachusetts

Received December 15, 1969

Summary

The three larger CNBr peptides from the α2 chain of chick skin collagen, α2-CB3, α2-CB4, α2-CB5 and a peptide with a molecular weight of about 60,000 containing an uncleaved methionyl residue, α2-CB(3-5), were separately renatured and aggregated into SLS crystallites for electron microscopy. Comparison of the resultant cross-striation patterns with those of the renatured α2 chains plus previously determined positions of two of the three smaller peptides by chemical means indicates the order of the CNBr peptides to be α2-CB(1-0-4-2-3-5). This coincides with that determined by chemical and isotope labelling techniques described in an accompanying paper.

Recently several laboratories (1, 2, 3) have renatured complete triple helical collagen-like molecules from the isolated α1 chain, $(\alpha 1)_3$, the α2 chain $(\alpha 2)_3$, and in the case of codfish skin collagen the α3 chain, $(\alpha 3)_3$(4). Moreover, several laboratories have reported successful renaturation of polypeptide fragments derived from collagen. Sakai and Gross (5) described the formation of SLS (Segment Long Spacing) fragments from the tadpole collagenase reaction products following denaturation and renaturation of the products, and Kühn et al. (6, 7, 8, 9) reported the renaturation of the

---

*This is publication #494 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities. This investigation was supported by grants from the National Institutes of Health (AM 3564 and AM 12785), and the RGK Foundation.

**Senior Investigator of the Arthritis Foundation at the Massachusetts General Hospital, Boston, Massachusetts

polypeptide fragments obtained by limited digestion of collagen with bacterial collagenase and trypsin. Rauterberg and Kühn (10) and Piez et al. (11) renatured some of the CNBr peptides from the α1 chain, and succeeded in forming the SLS aggregates from them. On the basis of these and other chemical data Piez et al. (11, 12) were able to order the CNBr peptides of the α1 chain of rat skin collagen.

We have applied a similar approach to the ordering of the CNBr peptides of the α2 chain of chick skin collagen. Results obtained from electron microscopic studies of the SLS aggregates of renatured α2-CB3, α2-CB4 and α2-CB5 form the substance of this report. Separation and characterization of the CNBr peptides from the α2 chain of chick skin collagen were recently described (13).

Material and Methods

Neutral salt-extracted and acid-extracted collagen were prepared from the skin of three-week-old normal and lathyritic chicks (14). The α2 chain was isolated by carboxymethyl (CM) cellulose chromatography (15), and was digested with CNBr according to the procedure described by Bornstein and Piez (16). The CNBr peptides were separated and purified by a combination of ion-exchange chromatography on phosphocellulose and CM cellulose, and molecular sieve chromatography on agarose (13). Renaturation of the CNBr peptides was accomplished by a procedure similar to that described by Rauterberg and Kühn (10). Purified α2, α2-CB3, α2-CB4, α2-CB5 and a peptide with a molecular weight of about 60,000 containing an uncleaved methionyl residue after CNBr reaction (13) were dissolved in 0.225 M citrate buffer, pH 3.7. The concentration of the peptide solution was 0.1 to 0.2%. Solutions of each peptide were heated at 45° for 10 minutes to destroy any preformed structure, and then the temperature was lowered in a stepwise

manner until 5° was reached. The incubation was continued for about 200 to 500 hours. The process of renaturation was monitored by determination of the reduced viscosity and the optical rotation. Viscometry was performed in a Cannon-Manning semi-micro type viscometer with a flow time of 89 seconds for the citrate buffer at 20°. The optical rotation was measured in a Rudolph spectropolarimeter with a mercury lamp at 365 mμ in a jacketed one decimeter cell. Renatured peptide solutions were dialyzed against 0.1 M acetic acid in the cold, and then dialyzed against a 0.4% solution of ATP (adenosine triphosphoric acid in the acid form) to aggregate the renatured peptides into the specifically banded SLS crystallites for electron microscopy. The SLS preparations were stained either with 0.5% solution of uranyl acetate in water or with a 1% solution of phosphotungstic acid (PTA) in 0.1 M sodium acetate, pH 4.2, and at times both types of staining were combined. All these procedures were performed at 5°. Photographs were taken with an RCA EMU 3G electron microscope.

Results and Discussion

The beginning temperature of renaturation, the specific optical rotation and the reduced viscosity at the end of renaturation of α2 and for the three CNBr peptides are shown in Table I. The cross-striation pattern of α2 SLS was similar to that of the native collagen SLS by visual observation. The renatured CNBr peptides nearly always formed dimers on renaturation and aggregation with ATP. These dimers were always antiparallel giving a symmetrical appearance. SLS aggregates of each of the CNBr peptides showed unique cross-striation patterns, allowing the location of each peptide along the length of the α2 chain by comparing the cross-striation pattern with that of α2. Electron micrographs of the SLS aggregates of each of the three CNBr peptides and a peptide containing one uncleaved methionyl

Table I

Renaturation Characteristics of CNBr Peptides of Chick Skin Collagen (Stepwise Cooling)

Peptide	Conc.	Ti*	Duration (hours)	ηrel	$-[\alpha]_{365}$
α2	0.1%	19°	197 hours	6.67	
α2-CB3	0.2%	15°	508	3.85	905 at 5°
α2-CB4	0.2%	20°	460	2.73	900 at 12°
α2-CB5	0.2%	21°	504	2.41	785 at 10°

Ti* = temperature of initial increase in $-[\alpha]_{365}$

residue along with those of the renatured α2 chain are presented in Figure 1. α2-CB4 is located near the "A" (NH_2-terminal) end of the α2 chain, occupying about 900 Å. Distal to α2-CB4 is a void of about 100 Å in length. Following the void is located α2-CB3, about 850 Å in length. Immediately abutting α2-CB3 is α2-CB5, 900 Å long, constituting the "B" (COOH-terminal) end. The latter finding is in agreement with the previous conclusion that α2-CB5 is the COOH-terminus of the α2 chain since it contains no homoserine (13). The peptide with molecular weight of 60,000 containing one uncleaved methionyl residue has the banded structure of α2-CB(3-5) and thus must be the uncleaved polypeptide of α2-CB3 plus α2-CB5.

Evidence that α2-CB1, a pentadecapeptide, is the NH_2-terminal peptide of the α2 chain was reported previously (17). The next in order is α2-CB0, a tripeptide, since an uncleaved dimer peptide of α2-CB1 and α2-CB0 joined by a methionyl residue was isolated by Vuust et al. (see the accompanying article). The position of the remaining peptide, α2-CB2, can be deduced to

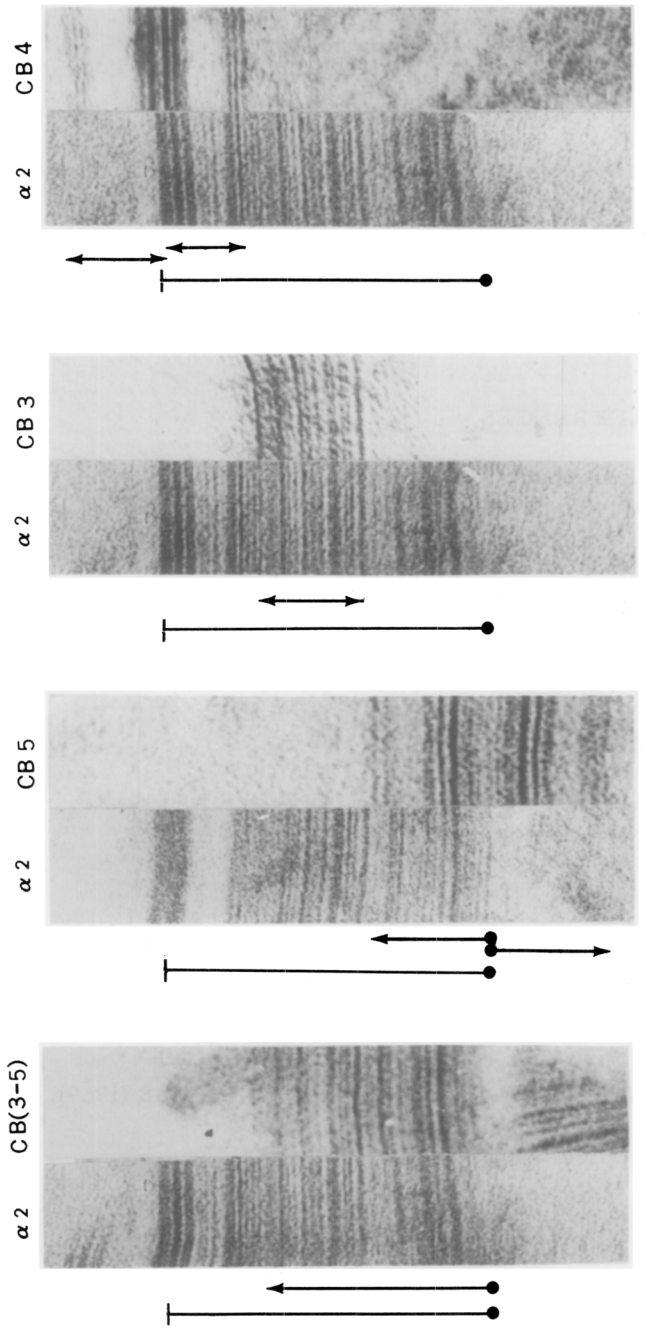

Figure 1. Electron micrographs of the SLS crystallites prepared from the renatured α2-CB (3-5), α2-CB5, α2-CB3 and α2-CB4 are matched with respect to band pattern with α2 SLS. The lines on the left side of each set of the micrographs are diagramatic representations of the relative locations of the CNBr fragments. The order of the CNBr peptides is diagramatically summarized at the bottom of the figure. The arrow-heads represent the cleaved free ends of the peptides, the solid circle at one end of the line represents the intact COOH-terminus of α2 and the perpendicular short line at the other end represents the intact NH_2 terminus.

be between α2-CB4 and α2-CB3, since this is the only empty space available, and corresponds to a fragment of 30 residues which is about 85 Å in length. On the basis of these observations it is suggested that the order of the CNBr peptides in the α2 chain of chick skin collagen is α2-CB (1-0-4-2-3-5). An identical conclusion was reached with regard to the order of the α2 CNBr peptides of rat bone collagen based on chemical and isotope labelling experiments described in an accompanying paper by Vuust et al.

REFERENCES

1. Tkocz, C., and Kühn, K., European J. Biochem., 7, 454 (1969).
2. Kühn, K., Tkocz, C., Zimmerman, B., and Beier, C., Nature, 208, 685 (1965).
3. Olsen, B.R., J. Ultrastruct. Res., 19, 432 (1967).
4. Olsen, B.R., J. Ultrastruct. Res., 19, 446 (1967).
5. Sakai, T., and Gross, J., Biochemistry, 6, 518 (1967).
6. Stark, M., and Kühn, K., European J. Biochem., 6, 542 (1968).
7. Muller, P., and Kühn, K., FEBS Letters, 1, 233 (1969).
8. Stark, M., and Kühn, K., European J. Biochem., 6, 534 (1968).
9. Kühn, K., and Eggl, M., Biochem. Z., 346, 197 (1966).
10. Rauterberg, J., and Kühn, K., FEBS Letters, 1, 230 (1969).
11. Piez, K.A., Bladen, H.A., Lane, J.M., Miller, E.J., Bornstein, P., Butler, W.T., Kang, A.H., Brookhaven Symposia in Biology, 21, 345 (1969).
12. Piez, K.A., Miller, E.J., Lane, J.M., and Butler, W.T., BBRC, 37, 801 (1969).
13. Kang, A.H., Igarashi, S., and Gross, J., Biochemistry, 8, 3200 (1969).
14. Kang, A.H., Piez, K.A., and Gross, J., Biochemistry, 8, 1506 (1969).
15. Piez, K.A., Eigner, E.A., and Lewis, M.S., Biochemistry, 2, 58 (1963).
16. Bornstein, P., and Piez, K.A., Biochemistry, 5, 340 (1966).
17. Kang, A.H., Piez, K.A., and Gross, J., Biochemistry, 8, 3648 (1969).