STRUCTURAL REQUIREMENTS OF ANTIGENIC DETERMINANTS IN THE AMINOTERMINAL REGION OF THE RAT COLLAGEN α2 CHAIN

Heinz Furthmayr, and Rupert Timpl *

Max-Planck-Institut für Eiweiß- und Lederforschung, Abt.Kühn, München, Germany

Received April 21, 1972

SUMMARY: Three overlapping antigenic determinants were defined by the use of several rabbit antisera to soluble rat collagen on the CNBr peptide α2-CB1 containing 14 amino acid residues. In each antigenic determinant the sequence ^{5}Lys-Gly was essential as well as, with one exception, the participation of a closely located tyrosyl residue. The minimal size involved four to six amino acid residues.

The immune response in the rabbit to a distinct antigenic determinant on rat collagen is characterized by the late appearance of antibodies during a hyperimmunization course (1). A detailed knowledge of the structure of this antigenic determinant should be valuable for further studies on the mechanism of such a phenomenon. The determinant group has been located on the cyanogen bromide (CNBr) peptide α2-CB1 originating from the aminoterminal end of the rat collagen α2-chain (1,2). Kang et al. (3) have elucidated the sequence of this peptide which contains 14 amino acid residues. However, it is rather unlikely that the entire sequence is necessary for the antigenic activity. Previously, aminoterminal antigenic determinants in the α1-chains of calf and human collagen were found to have a requirement of 5-10 amino acid residues only (4). In the present study this question was investigated for the rat collagen peptide using

* Supported by a grant of the Deutsche Forschungsgemeinschaft, SFB 37.

proteolytic fragments in a serologic inhibition assay.

METHODS: The peptide α2-CB1 was isolated from the rat collagen α2-chain after cleavage with CNBr (5). A small amount of α2-CB1^{Ald} which contains α-aminoadipic-δ-semialdehyde instead of lysine was purified by chromatography on phosphocellulose (5,6). The amino acid composition was determined as described (7).

For fragmentation trypsin (2xcryst., Worthington, Freehold, N.J., treated with L-(1-tosylamido-2phenyl)ethyl chloromethyl ketone) was applied at an enzyme-substrate ratio 1:100 in 0.2 M ammonium bicarbonate pH 7.8 for 4 hours at 37°C. Treatments with α-chymotrypsin (48 units/mg, Worthington), with elastase (2xcryst., Serva, Heidelberg) and with thermolysin (6000 PU/mg, Merck, Darmstadt) were carried out as described recently (4). Each digestion was terminated by addition of acetic acid followed by lyophilization. The digests (1-5 mg) were chromatographed at room temperature on a Bio-Gel P-4 column (115x1,5 cm) which was equilibrated with 0.1 M acetic acid. The tryptic peptides were separated by paper electrophoresis in 0.25 M pyridine acetate pH 5.4 at 50 V/cm for 2 hours.

Rabbit antibodies entirely specific for rat collagen α2-CB1 were obtained from six hyperimmune animals upon immunoadsorption (1). In this study also two early immune sera (see ref.1) obtained after immunization with native (No.317) or with denatured rat collagen (No.434) were included. The antibody solutions were titrated in passive hemagglutination vs. red cells coated with rat collagen α2-chains and revealed titers between 1:32 and 1:512. For the inhibition assay the antibody solutions were diluted to 8 agglutinating units in phosphate-buffered saline pH 7.2. These dilutions were mixed with equal volumes of inhibitor solution of different concentrations (4). After

addition of α2-chain-coated red cells the lowest inhibitor concentration completely preventing agglutination was recorded.

Table I

Amino Acid Composition of Rat Collagen α2-CB1 and Its Proteolytic Fragments [a)]

	α2-CB1	C1	C2	Th1	Th2	E1	E2
aspartic acid	1.0	-	1.0	1.0	-	1.0	-
serine	1.9	-	1.8	1.1	1.2	1.0	0.8
homoserine	1.0	-	1.0	-	1.0	-	1.0
glutamic acid	1.0	1.1	-	0.9	-	1.1	-
proline	2.1	-	2.0	-	1.9	-	2.0
glycine	3.3	-	3.0	1.1	2.3	0.9	2.3
alanine	1.2	-	1.0	-	1.0	-	0.9
valine	1.0	-	1.0	-	1.0	0.7	-
tyrosine	0.8	0.9	-	0.6	-	0.9	-
lysine	1.0	-	0.9	0.8	-	1.0	-
total [b)]	14	2	12	6	8	7	7
position	1-14	1-2	3-14	1-6	7-14	1-7	8-14

a) Given as residues/peptide. A dash denotes values below 0.1. The abbreviations designate chymotryptic (C), thermolysin (Th) or elastase (E) peptides.
b) Calculated on the basis of the nearestwhole number.

RESULTS: The amino acid composition of α2-CB1 and of its proteolytic fragments is given in Table I. The amino acid sequence as well as the positions of enzymatic cleavage are outlined in Fig.1. The two peptides obtained after digestion with thermolysin or chymotrypsin, respectively, could be purified by chromatography on Bio-Gel P4 (Fig.2 and ref.3). Elastase cleaved mainly between ^{7}Val-Ser. The yield of the peptide E1 (position 1-7) depended on the enzyme-substrate ratio as well as on the time of incubation. Some preparations were obtained which had considerably decreased contents of valine and glycine suggesting the action of carboxypeptidases which might contaminate elastase. The other fragment E2 could be sufficiently purified from uncleaved material on Bio-Gel P-4 (Fig.2). Trypsin treatment rendered two peptides

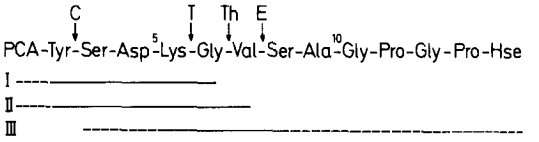

Fig.1: Sequence of rat collagen α2-CB1 (ref.3) and correlation of three antigenic determinants I-III. The full line characterizes the minimal size, the dashed line denotes residues the involvement of which could not be established. At the top the cleavage points of chymotrypsin (C), elastase (E), trypsin (T) and thermolysin (Th) are indicated by arrows.

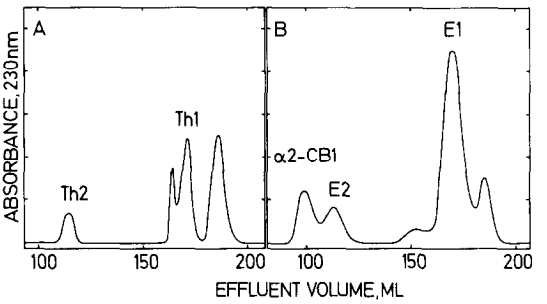

Fig.2: Chromatography on a Bio-Gel P-4 column (115x1,5 cm) of peptides obtained after thermolysin (A) or elastase (B) treatment of rat collagen α2-CB1. Peaks not designated contained salt and/or traces of amino acids only.

T1 (position 1-5) and T2 (position 6-14) separable by high voltage electrophoresis. The amino acid composition corresponded to the sequence data.

Antibodies specific for α2-CB1 obtained from eight individual rabbits were studied by hemagglutination-inhibition (Table II). Each antiserum could be clearly inhibited by low concentrations of α2-CB1. Comparison with α2-CB1^{Ald} revealed highly variable results. Some antisera (e.g.No.290) could only be inhibited by 60fold higher amounts of α2-CB1^{Ald}, others (No.354) showed no difference between both peptides within the limits of experimental error ($\pm$ 50%).

The inhibition experiments with the proteolytic fragments demonstrated even more striking differences. One antiserum

Table II

Minimum Amounts (μmoles/l) of α2-CB1 or Proteolytic Fragments Required to Inhibit 8 Agglutinating Units of Rabbit Antibodies Specific for Rat Collagen α2-CB1

Inhibitor [a]	Antiserum No. 290	317	288	351	354
α2-CB1	0.025	0.1	0.1	0.025	0.05
α2-CB1^{Ald}	1.6	0.4	1.6	0.2	0.05
α2-CB1 T1+2 [b]	>12.8	>12.8	>12.8	>12.8	12.8
" Th1	0.025	>12.8	>12.8	>12.8	1.6
" Th2	>12.8	>12.8	>12.8	>12.8	>12.8
" C1	>12.8	>12.8	>12.8	>12.8	>12.8
" C2	>12.8	0.05	>12.8	0.8	>12.8
" E1	0.05	>12.8	0.2	0.1	0.05
" E2	0.8	6.4	12.8	1.6	1.6

a) See Table I for explanation of the fragments.
b) Unfractionated tryptic peptides.

(No.290) could be effectively inhibited by the thermolysin peptide Th1 (position 1-6) and the elastase peptide E1 (position 1-7). None of the other peptides were active. This antiserum was obtained after 10 and 16 antigen injections (1) and revealed no change in specificity during this three months period. The other extreme is represented by the early immune serum No.317 which could be inhibited only by the chymotryptic peptide C2 (position 3-14). The remaining six antisera, including a second early responder serum, for which representative examples are given could be effectively inhibited only by the peptide E1. They differ from each other to some extent with regard to decreased activities found in Th1 (No.354) or C2 (No.351). Most antisera showed additionally a low activity for E2, however, not comparable to that found for α2-CB1. It is likely that this activity reflects contamination by a small amount of uncleaved α2-CB1, as

suggested from the chromatographic position (Fig.2), which is not detectable by amino acid analysis. None of the antisera showed activity for the tryptic peptides.

DISCUSSION: In Fig.1 the sequence of rat collagen α2-CB1 is correlated with the serologic results. At least three different antigenic determinants were revealed on this small peptide by the use of individual antisera. These determinants overlap each other in the region ^{5}Lys-Gly as concluded from the complete loss of activity observed after trypsin digestion. Even a modification of the ε-amino group of lysine by oxidation to an aldehyde group had a strong effect for some of the antisera. Since this aldehyde is of functional importance for the cross-linking reaction (8) one should assume that the antigenic properties of cross-linked rat collagen are also modified.

The use of different fragments allowed a more concise localization. One antigenic determinant (type I, antiserum No.290) is confined to the sequence positions 1-6 though the involvement of the PCA residue (pyrrolidone-5-carboxylic acid) is not certain. It was surprising that the glycyl residue in position 6 was essential for the activity. An analogous example has been found in human collagen (4). Most of the antisera required additionally the valine in position 7 (type II determinant). A third antigenic determinant (type III, antiserum No.317) did not involve the tyrosine in position 2 but a sequence exceeding beyond the valine. This antiserum was obtained from a rare early responder (1). It is tempting to speculate that the different sequence requirement bears a relation to the responder state. However, a second early responder had antibodies specific for the type II determinant.

The maximal size of the antigenic determinants I and II are

correlates with that of hepatic enzymes and smooth endoplasmic reticulum. This is of interest since the polymerase II enzyme is believed to synthesize RNA which translates, at least in part, for the enzymes and other "non-ribosomal" genes. It is apparent that a correlation exists between the diurnal variation in each of the endogenous RNA polymerases and certain specific biochemical processes (Fig. 2).

Finally one must inquire if these daily variations are of academic interest only or must they be considered in protocol design? When the hormonal induction of polymerase activity is reported, do the data actually reflect an action of the hormone on the enzyme or is the rise which is assigned to the hormone fortuitous but actually due to the daily rhythmicity in the enzyme's synthesis and activity? Thus, even when proper controls are included, animals assayed during periods of peak polymerase activity (either I or II) may not be as responsive to various stimuli as are animals used during the trough periods of enzyme activity.

ACKNOWLEDGMENTS

The authors wish to extend their appreciation to Mrs. Estella Aughtrey, Mrs. Gayle Cashion, Miss Virginia Lukas and Miss Sherri Willis for their excellent assistance and to Dr. Frank Chytil for his review of this manuscript. Thanks are also due Professor T. Wieland (Max Planck Institute, Heidelberg) for his generous gift of α-amanitin.

Supported by USPHS CA-13065-01, Ford Foundation 630-0141A and the Vanderbilt Center for Population Research (USPHS HD-05797).

REFERENCES

1. Black, I.B. and Axelrod, J. in Biochemical Actions of Hormones, G. Litwack, Ed., Academic Press, N.Y. (1970), p. 135.
2. Barbiroli, B. and Potter, V.R. Science 172, 738, 1971.
3. Barnum, C.P., Jardetzky, C.D. and Halberg, F. Am. J. Physiol. 195, 301, 1958.
4. Jaffee, J.J. Anat. Rec. 120, 935, 1954.
5. Chedid, A. and Nair, V. Science 175, 176, 1972.
6. Rapport, M.I., Feigin, R.D., Burton, J. and Beisel, W.R. Science 153, 1642, 1966.
7. Civen, M., Ulrich, R., Trimmer, B.M. and Brown, C.B. Science 157, 1563, 1967.
8. Radzealowski, F.M. and Bousquet, J. Pharmacol. Exp. Therap. 163, 229, 1968.
9. Colas, A., Gregonis, D. and Moir, N. Endocrinology 84, 165, 1969.
10. Aschoff, J. Naturwissenschaften, 38, 506, 1951.