AMINO ACID SEQUENCE NEAR THE AMINO TERMINUS OF PORCINE PEPSIN[1]

Jordan Tang[2]

Genetic Institute, Department II, University of Copenhagen, Copenhagen, Denmark

Received September 29, 1970

Summary: The amino acid sequence near the amino terminus of porcine pepsin was studied. By diagonal electrophoresis (1), a peptide was isolated containing 9 residues from the amino terminus of pepsin. Structural studies established their sequence to be Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr. Comparison of this amino-terminal sequence with that known for rennin showed a definite homology. Alignment of the homologous residues revealed that the sites at which the respective zymogens are cleaved to form active pepsin and rennin are 2 residues apart. The implications of this finding in the mechanism of activation of zymogens of the acidic proteases are discussed.

The existence of a structural homology among certain gastric proteases, including pepsin, rennin, and gastricsin is now well established (2, 3). Apparently, these enzymes descended from a common ancestral protease during their evolution. Porcine pepsin and bovine rennin, in particular, are closely homologous in structure, as evidenced by comparison of partial amino acid sequences in the two enzymes (4). In contrast to the common homology shared elsewhere in these molecules, however, the first 5 residues from the amino termini of pepsin and rennin are completely dissimilar. In rennin, the sequence is Gly-Glu-Val-Ala-Ser (5), whereas in pepsin it is Ile-Gly-Asp-Glu-Pro (6). The disparity is especially surprising when one considers that each enzyme is activated from its respective zymogen by hydrolysis of a peptide bond to

---

[1]This work was partially supported by NIH Research Grant AM-01107.

[2]Recipient of NIH Career Development Award 5 KO4-AM-38638. Permanent address: Oklahoma Medical Research Foundation, University of Oklahoma Medical Center, Oklahoma City, Oklahoma 73104.

form a new amino terminus in the active enzyme. One would thus expect the sequences near the amino termini of these enzymes to be more homologous, as a reflection of limited evolutionary change.

Previous work on pepsin has identified a sequence of only 5 residues from the amino terminus (6), as opposed to a much longer amino-terminal sequence for rennin (5). Since it was felt that comparison of a longer sequence in pepsin with that already known for rennin would prove revealing, studies were undertaken to establish in further detail the amino acid sequence near the amino terminus of pepsin. The method of diagonal electrophoresis of Butler et al. (1) was adopted because the techniques involved (described below) permit isolation of specific amino-terminal peptides.

Materials and Methods: Crystalline pepsin was obtained from Sigma Chemical Co., St. Louis, Mo. Thermolysin was purchased from Daiwa Kaseai K. C. Co., Osaka, Japan. Subtilisin was purchased from Novo Terapeutisk Laboratorium, Copenhagen, Denmark. Other chemicals used were of the highest purity purchased from commercial sources.

The amino groups on the protein (such as the amino-terminal group and the ε-amino groups of lysine and aminoethyl cystein) were reacted with maleic anhydride to form maleyl derivatives according to the method of Butler et al. (1). Thirty times molar excess of maleic anhydride powder was added in small portions to a 1% solution of protein, and the pH was maintained between 8 and 9 by the addition of Na_2CO_3 powder. Within 10 minutes at room temperature, maleylation was complete. After dialysis against 0.05 M NH_4OH, the protein was lyophilized. The subtilisin digestion was carried out by adding 1 mg of subtilisin to 100 mg of maleylated protein in 5 ml of 0.2 M N-ethylmorpholine acetate buffer, pH 8.0, and incubating the mixture for 16 hours at 37°C.

Detailed procedures in diagonal electrophoresis were performed as previously described (7, 8). After proteolysis and first-dimensional electrophoresis, the masking

maleyl group was removed, thus altering the electrophoretic mobility of the involved peptides in the 2nd-dimensional electrophoresis. Removal of the maleyl group was accomplished by exposing the paper strips to the vapor of a pyridine acetate buffer, pH 3.5, for 18 hours at 60° (1).

The Edman sequential degradation was carried out by determination of the newly formed amino-terminal residue (9). The dansyl amino acids were determined by thin-layer chromatography on polyamide sheets (10). Carboxyl-terminal residues were identified by carboxypeptidase digestion (11).

Results: Diagonal electrophoresis of the subtilisin digest of maleyl porcine pepsin produced a pattern (Fig. 1, Part A) in which the major spot laying off the diagonal line was peptide NT. This peptide was assumed to have derived from the amino terminus of pepsin, as judged from its mobility change in the two dimensions. The peptide spots which lay above or near the neutral line of the second dimension (L_1-L_9) were assumed to be lysine-containing peptides derived from carboxyl-terminal region of the molecule

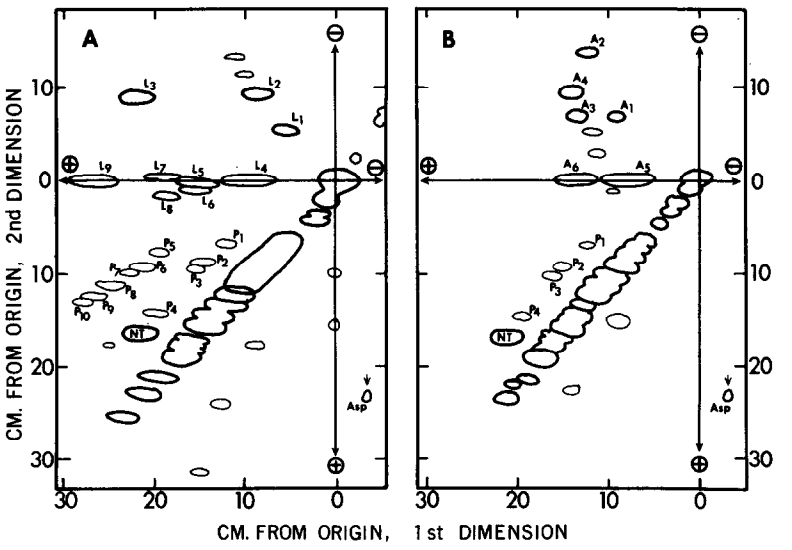

Fig. 1. Diagrams of pH 6.5 diagonal electrophoresis of demaleylated and subtilisin digest of porcine pepsin (A) and a cyanogen bromide fragment derived from the amino-terminal half of reduced and aminoethylated porcine pepsin (B).

(12). Deamidation during heating was presumed to be responsible for the few peptide spots that appeared below the diagonal line. Numerous spots of minor intensity (P_1-P_{10}) were found near the diagonal line. Since Rajagopolan et al. (13) showed that commercial crystalline pepsin contains many autolytic cleavage sites, peptides P_1-P_{10} were assumed to be the amino-terminal peptides produced from these autolytic sites. To confirm this analysis, maleylation and diagonal electrophoresis experiments were performed on a purified fragment produced by cyanogen bromide cleavage of reduced and aminoethylated pepsin. Fragment CB_2, which contained about 160 residues, was derived from the amino-terminal half of the pepsin molecule (Tang, unpublished results). Peptide NT again emerged as the major spot laying off the diagonal (Fig. 1, Part B). Only traces of peptides P_1-P_4 were present. Peptides A_1-A_6 were obviously peptides containing aminoethyl cystein, the sequence of which is known (4).

These experiments made it evident that peptide NT, the only major peptide present in both diagonal electrophoretic patterns, must have derived from the amino terminus of pepsin. Peptide NT was purified from 100 mg of pepsin, with a molar yield of 12.5%. The amino acid analysis showed it to contain 9 residues (Table I). Its amino-terminal residue, Ile, was the same as that of the whole protein. The carboxyl-terminal residue was Tyr. Peptide NT digested with thermolysin at a substrate-enzyme ratio of 50:1 at 37° C for 18 hours gave two peptide bands on paper electrophoresis at pH 6.5. Peptide NT_1 contained 5 residues and had an amino-terminal Ile, apparently derived from the amino-terminal portion of peptide NT. By using the Edman degradation and dansyl procedures, the sequence was established to be Ile-Gly-Asp-Glu-Pro. Peptide NT_2 contained 4 residues, with the sequence Leu-Glu-Asn-Tyr. The acids and amides were determined by the mobility of the peptides at different steps of Edman degradation (Table I). Thus, the sequence of peptide NT was established to be: Ile-Gly-Asp-Glu-Pro-Leu-Glu-Asn-Tyr.

TABLE I

Amino Acid Composition and Characteristics of
Peptides from Amino-Terminal Region of Pepsin

Peptide	Electr. Mobility in pH 6.5[1]		Cadmium - Ninhydrin Color	Amino Acid Composition (molar ratio)						
	1st Dim.	2nd Dim.		Asp	Glu	Pro	Gly	Ile	Leu	Tyr
NT	-1.05	-0.73	Red (S)	1.97	2.04	1.01	1.00	0.98	1.18	0.96
NT_1		-0.84	Red (S)	1.22	1.08	1.10	1.00	0.96	-	-
NT_2		-0.38	Orange — Red	1.07	1.00	-	-	-	1.01	0.70
Peptides from Edman Degradation[3]:										
NT_1-2		-1.05	Orange							
NT_1-3		-0.69	Red							
NT_2-1		-0.47	Red							
NT_2-2		0.09[4]	Orange							

1. The electrophoretic mobilities are relative to that of Asp (= -1.0).
2. The sign (S) designates that color development was slow.
3. The numbers after the - sign indicate the number of residues removed in Edman degradation.
4. Slight mobility is associated with an asparagine amino terminus; the peptide is considered neutral.

Discussion: Peptide NT is apparently derived from the 9 amino-terminal residues of porcine pepsin. The sequence of NT agrees with that reported by Stepanov (6) for the first 5 residues. However, with 9 residues now established, a homology with the amino-terminal sequence of rennin (5) becomes clear, as shown in Fig. 2. It is interesting that rennin contains two more residues at the amino terminus when the homologous residues are aligned, evidently as a result of the difference in cleavage sites during activation of the respective zymogens. However, the homology extends into the last two residues in the peptide portion of pepsinogen (-Ala-Glu, residue 40 and 41 in pepsinogen). Ong and Perlmann (14) showed that the peptide bond which is broken during activation of pepsinogen is a Glu-Ile bond. The corresponding position in rennin is a closely analogous Glu-Val bond. However, the activation of pro-rennin is accompanied by hydrolysis of an X-Gly bond two residues away, a somewhat surprising finding since the specificities of pepsin and rennin in proteolysis are very similar. The sites on the B-chain

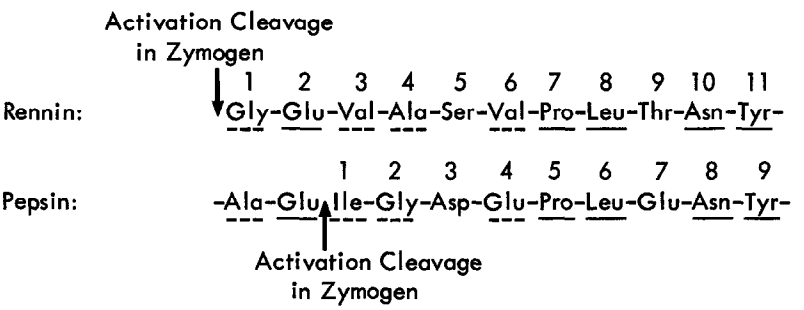

Fig. 2. Homology in amino acid sequence of the amino-terminal regions in bovine rennin (5) and porcine pepsin. The solid underlines indicate the residues which are identical; the broken underlines indicate the residues whose difference could be the result of a single-step mutation. The numbering of amino acid residues, in both cases, starts at the amino terminus of the enzymes. The two residues on the left of amino terminus of pepsin (-Ala-Glu) are the last two residues in the portion of 41 residues which are cleaved off during the activation of pepsinogen (14).

of oxidized insulin that are hydrolyzed by pepsin (15) and rennin (16) are nearly identical. Were the hydrolytic activity of pepsin and rennin responsible for hydrolysis of the bonds discussed above during zymogen activation, the cleavage sites would be limited by the conformation of the zymogen. On the other hand, it seems possible that the activation of these zymogens may be independent of the hydrolytic activities of the enzymes. Cleavage of a specific site might be triggered by change of the ionization state of a group with a pK of about 5, thus producing an internal rearrangement of bonds. This alternative is especially plausible when one considers two other factors: first, that the hydrolyzed bond Glu-Ile in the activation of pepsinogen is relatively unfavorable for the specificity of the enzyme (17, 18), and second, that the activation of pepsinogen and pro-rennin requires the presence of no preformed enzyme.

Acknowledgement: The author wishes to thank Professor Bent Foltmann, Genetic Institute, II, University of Copenhagen, for the helpful discussions and for the use of facilities during the course of this work.

REFERENCES

(1) Butler, P.J.G., Harris, J.I., Hartley, B. S., and Leberman, R., Biochem., J., 112 : 679 (1969).
(2) Foltmann, B. and Hartley, B.S., Biochem. J., 104 : 1064 (1967).
(3) Huang, W.Y. and Tang, J., J. Biol. Chem., 245 : 2189 (1970).
(4) Tang, J. and Hartley, B.S., Biochem. J., 118 : 611 (1970).
(5) Foltmann, B., Phil. Trans. Roy. Soc. London, B 257 : 147 (1970).
(6) Stepanov, V.M., Abstr. Fed. Europ. Biochem. Soc., 5th Meeting, No. 1089, (1968).
(7) Brown, J.R. and Hartley, B.S., Biochem. J., 101 : 214 (1966).
(8) Tang, J. and Hartley, B.S., Biochem. J., 102 : 593 (1967).
(9) Gray, W.R., Methods in Enzymology, Vol. II, p. 469, Ed. Hirs, C.H.W., N.Y. Acad. Press, Inc. (1967).
(10) Woods, K.R. and Wang, K.T., Biochim. Biophys. Acta, 133 : 369 (1967).
(11) Ambler, R.P., Methods in Enzymology, Vol. II, p. 417, Ed. by Hirs, C.H.W., N.Y. Acad. Press, Inc. (1967).
(12) Dopheide, T.A.A., Moore, S., and Stein, W.H., J. Biol. Chem., 242 : 1833 (1967).
(13) Rajagopalan, T.G., Stein, W.H., and Moore, S., J. Biol. Chem., 241 : 4940 (1966).
(14) Ong, E.B. and Perlmann, G.E., J. Biol. Chem., 243 : 6104 (1968).
(15) Sanger, F. and Tuppy, H., Biochem. J., 49 : 481 (1951).
(16) Foltmann, B., Compt. Rend. Trav. Lab. Carlsberg, 35 : 143 (1966).
(17) Tang, J., Nature, 199 : 1094 (1963).
(18) Fruton, J.S., Symposium on Structure and Function Relationships of Proteolytic Enzymes, p. 222, Ed. Desnuelle, P., Neurath, H. and Ottesen, M., Munksgaard, Copenhagen, Denmark (1970).