ACTIVE DERIVATIVES OF A POLYPEPTIDE INHIBITOR OF TRYPSIN (KUNITZ AND NORTHROP INHIBITOR)

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The pancreatic inhibitor of trypsin, known under the name of Kunitz and Northrop inhibitor, involves a single polypeptide chain of 58 amino acid residues the primary structure of which has been determined (Chauvet et al., 1964, 1966 a, b ; Dlouhá et al., 1965 ; Kassel et al., 1965 a, b). The mechanism of action of this inhibitor on trypsin has been the object of detailed research (Kunitz and Northrop 1936 ; Grob, 1949 ; Green, 1953 ; Green and Work, 1953) but there is still not very much known on the chemical groups of the molecule which intervene during the union with the enzyme. Green and Work (1953) taking into consideration the pH of total dissociation of the inhibitor-trypsin complex deduced that an arginine residue was involved in the bonding. This would imply that at least one of the sites participating in the blocking has characteristics of a substrate. Since the N-terminal sequence Arg-Pro might correspond to this particular site, a series of experiments has been undertaken to determine the possible role of the N-terminal VS other residues of the inhibitor.

I - Guanidination of the inhibitor

The guanidination of proteins by means of O-methyl isourea generally leads to selective substitution of the ε-amino groups of the lysine residues, while the reaction does not affect the α-amino group or groups (Chervenka and Wilcox, 1956). According to the results obtained with various hormones and enzymes (Evans and Saroff, 1957 ; Geschwind and Li, 1957 ; Hettinger and Harbury, 1965), the transformation of lysine

residues into homoarginine residues does not appear to have an effect on the biological activity.

The conditions used are close to those employed by Hettinger and Harbury (1965) : 150 mg of inhibitor dissolved in 2 ml water are mixed with 3 ml of a molar solution of O-methyl isourea sulphate and the pH is adjusted to 11 with 3N NaOH. The volume is brought to 9 ml and the reaction is allowed to take place over 6 days at 4°C. The pH is then brought to 3 with acetic acid, the solution is concentrated to 2 ml and the guanidine derivative is freed from salts and excess reagent by passing through a column of Sephadex G-25 (1,5 x 80 cm) equilibrated with 0,1 N acetic acid. The presence of the derivative in the fractions is revealed by its absorption at 280 mμ ; the fractions containing the product are pooled and the solution is lyophilized. 122 mg material are obtained in this way. Analysis for amino acids shows that the transformation of the 4 lysine residues which the molecule contains is virtually quantitative : the guanidinated derivative contains 3,87 homoarginine residues and 0,17 lysine residues per mole. On the other hand the α-amino group of the N-terminal arginine is not modified, since approximately equal amounts of dinitrophenyl-arginine are obtained when dinitro-fluorobenzene is caused to act either on the native or on the guanidinated inhibitor. The specific molecular activity of the guanidinated derivative is very slightly higher than that of the native inhibitor.

II - Blocking of the α-amino group of the guanidinated inhibitor

Since the fully active guanidinated inhibitor has only a single amino group it is possible to block this group selectively using conventional processes such as acetylation or succinylation. Acetylation was carried out according to Fraenkel-Conrat et al. (1949) under the conditions employed by Wong and Liener (1960) : 18 mg of guanidinated inhibitor dissolved in 1 ml water are mixed with 3 ml of a saturated solution of sodium acetate. 0,1 ml of acetic anhydride is added over 2 hours, then the solution is kept for 18 h at 0° with stirring. The elimination of salts is achieved by passing through a column of Amberlite MB_3 (2 x 30 cm). The solution obtained, when concentrated, contains the acetyl guanidino inhibitor. The blockage of the α-amino group, checked by reaction with dinitrofluoro-

benzene, is higher than 95 %. The specific molecular activity of the derivative is slightly higher than that of the native inhibitor. By contrast when the acetylation is carried out directly on the native inhibitor a 93 % reduction in activity is observed.

Succinylation of the guanidinated inhibitor has been carried out according to Habeeb et al. (1958) : to 20 mg of the product dissolved in 5 ml M phosphate buffer, pH 7,8, there is added 100 mg of succinic anhydride in small quantities during one hour. The reaction is allowed to take place over 24 hours at 4°C with stirring. The salts and the excess reagent are eliminated by passage through a column of Sephadex G-10 (0,9 x 80 cm. The solution containing the succinyl guanidino inhibitor is lyophilized. The blockage of the α-amino group, checked as described above, is higher than 95 %. The specific molecular activity of the derivative is approximately equal to that of the native inhibitor. The direct succinylation of the native inhibitor involves a 95 % reduction in the specific activity. The agreement of the results obtained either by acetylation or by succinylation indicate that the α-amino group of the inhibitor does not enter into the inhibition mechanism and further suggests that one or several of the lysine ε-NH_2 groups might be involved.

III - Shortening of the chain of the guanidinated inhibitor

The N-terminal arginine residue of the polypeptide chain has been detached using a modification of the Edman technique (Edman, 1960). 12,5 mg of guanidinated inhibitor are dissolved in 0,5 ml of 0,5 M ammonium bicarbonate. 0,06 ml of phenyl isothiocyanate is added and it is kept under stirring for 1 hour at 37°. The excess reagent is extracted by benzene and the aqueous phase is lyophilized. The operation is repeated a second time and the lyophilization yields 11 mg of phenylthiocarbamyl polypeptide. 1 mg is set aside for titrations and 10 mg are dissolved in 0,5 ml anhydrous trifluoracetic acid and the reaction is allowed to take place for 1/2 hour. The reagent is then eliminated by vacuum evaporation and the operation is repeated. The dry residue is finally dissolved in 0,5 ml of 0,1 N acetic acid and kept at 0° for 1 hour. The liberation of PTH-arginine is checked by paper electrophoresis. The material

is placed on a column of Sephadex G-25 (1,5 x 80 cm), it is washed with 0,1 N acetic acid and 3 ml fractions are collected. The fractions which contain the protein material are combined and concentrated to 2 ml. Analysis for amino acids indicates 4,91 arginine residues in place of 6 contained in the intact molecule. The biological activity of the truncated guanidinated inhibitor is 93 % of the intact guanidinated inhibitor.

IV - Progressive elongation of the chain of the guanidinated inhibitor

Katchalski et Sela (1958) have shown that it is possible to graft polyamino acids of variable length at the position of the amino groups of the proteins. It is logical to assume that when the ε-amino groups have been guanidinated, the grafting can only take place at the position of the α-amino group of the chain, i. e. at the N-terminal end of the chain. Since the elongation is produced by successive additions of residues, the growth of the chain can be controlled to a certain extent by the amount of reagent and the duration of the reaction.

20 mg of guanidinated inhibitor are dissolved in 5 ml of phosphate buffer 0,05 M at pH 7. The solution is kept in melting ice and 2 ml of dioxane containing 6 mg of N-carboxy-DL-alanine anhydride is added drop by drop. It is kept for 1 hour at 0° then the solution is lyophilized. 51 mg of material are obtained, which are dissolved in 3 ml 0,1 N acetic acid and passed through a column of Sephadex G-25 so as to eliminate the salts and the contaminating polyalanines. The fractions containing the polyalanyl guanidinated inhibitor are combined and the solution is lyophilized. It is observed on the one hand that the blocking of the α-amino group is complete (dinitrofluorobenzene technique) and on the other hand that the product retains 88 % of the specific molecular activity of the native inhibitor. The number of alanine residues fixed in the N-terminal position was determined as follows : the derivative, oxidized by performic acid, is split by trypsin and the fragments obtained are separated by the fingerprinting technique under the conditions described previously (Chauvet et al., 1966 b). The N-terminal peptide is isolated and analysed. 1,8 fixed alanine residues per mole are found. The other tryptic fragments correspond to those supplied by the tryptic hydrolysis of the oxidized gua-

nidinated derivative. It can thus be deduced that the fixation of an Ala-Ala peptide in the N-terminal position does not appear appreciably to modify the inhibiting capacity. The alanination of the guanidinated inhibitor was then carried out using 160 mg of anhydride instead of 6 mg and allowing the reaction to take place over 3 days instead of 1 hour. The other operations are identical. The blockage of the α-amino group is complete and the product has only 7 % of the initial activity. The N-terminal tryptic peptide is isolated as before and analysed. 12,5 fixed alanine residues per mole are found. It thus appears that the inhibitor capacity will not be changed if the alanine chain fixed in the N-terminal position has two residues but that it disappears when the length of the chain reaches 12-13 residues. It should be noted that the anhydride used is that of DL-alanine and that it would be of interest to check these results with L-alanine.

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References

Chauvet, J., Nouvel, G. and Acher, R., (1964), Biochim. Biophys. Acta, 92, 200.
Chauvet, J., Nouvel, G. and Acher, R., (1966 a), Biochim. Biophys. Acta, 115, 121.
Chauvet, J., Nouvel, G. and Acher, R., (1966 b), Biochim. Biophys. Acta, 115, 130.
Chervenka, C. H., and Wilcox, P. E., (1956), J. Biol. Chem., 222, 635.
Dlouhá, V., Pospisilova, D., Meloun, B. and Sorm, F., (1965), Coll. Czech. Chem. Comm., 30, 1311.
Edman, P., (1960), Ann. N. Y. Acad. Sc., 88, 602.
Evans, R. L., and Saroff, H. A., (1957), J. Biol. Chem., 228,295.
Fraenkel-Conrat, H., Bean, R. S. and Lineweaver, H., (1949), J. Biol. Chem., 117, 385.
Geschwind, I. I. and Li, C. H., (1957), Biochim. Biophys. Acta, 25, 178.
Green, M., (1953), J. Biol. Chem., 205, 535.
Green, M. and Work, T. S., (1953), Biochem. J., 54, 347.

Grob, D., (1949), J. Gen. Physiol., 33, 103.
Habeeb, A. F. S. A., Cassidy, H. G. and Singer, S. J., (1958), Biochim. Biophys. Acta, 29, 587.
Hettinger, T. P. and Harbury, H. A., (1965), Biochemistry, 4, 2585.
Kassel, B., Radicevic, M., Ansfield, M. J. and Laskowski, M. Sr., (1965), Biochem. Biophys. Res. Comm., 18, 255.
Kassel, B. and Laskowski, M. Sr., (1965), Biochem. Biophys. Res. Comm., 20, 463.
Katchalski, E. and Sela, M., (1958), Adv. in protein chemistry, 13, 243.
Kunitz, M. and Northrop, J. H., (1936), J. Gen. Physiol., 19,991.
Wong, R. C. and Liener, I. E., (1960), Biochim. Biophys. Acta, 38, 80.