ISOLATION OF ASPARTYL-PHENYLALANINE AMIDE FROM CHOLECYSTOKININ-PANCREOZYMIN[1)]

Viktor Mutt and J. Erik Jorpes

Chemistry Department and The Wallenberg Foundation Laboratory for Physiological Chemistry, Karolinska Institutet, Stockholm 60, Sweden

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The preparation of a polypeptide material exhibiting the biological activities previously ascribed to two different gastrointestinal hormones, cholecystokinin and pancreozymin, has recently been described (Jorpes et al., 1964). Work currently in progress in this laboratory indicates that material prepared in this manner is an essentially homogeneous polypeptide, the preliminary analysis of which showed the following amino acid composition, if amide groups are disregarded:

Ala_1, Arg_3, Asp_5, Glu_2, Gly_2, His_1, $Ileu_2$, Leu_2, Lys_2, Met_3, Phe_1 Pro_2, Ser_4, Tyr_1, Try_1, Val_1.

One of the two lysines is N-terminal in the chain. Cysteine, cystine and threonine are absent (Jorpes et al., 1964). It seemed to be of interest to ascertain how the polypeptide would behave on treatment with cyanogen bromide under the conditions worked out by Gross and Witkop (1960) for the cleavage of methionyl linkages. It was found that, although the picture obtained was quite complicated, the dipeptide aspartyl-phenylalanine amide could be isolated from the reaction pro-

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ducts, indicating that the polypeptide terminates in aspartyl-phenylalanine amide, and probably, because of the method used for fragmentation, in methionyl-aspartyl-phenylalanine amide. This C-terminal sequence is identical to that of another gastrointestinal hormone, gastrin (Gregory et al., 1964). It should also be mentioned that still another gastrointestinal hormone, secretin, terminates in an amide, the amide of valine (Mutt et al., 1965).

Another of the fragments obtained on cyanogen bromide degradation of cholecystokinin-pancreozymin shows that the tripeptide sequence •Gly•Try•Met• constitutes part of the polypeptide chain. Since evidence from degradation of the polypeptide with trypsin shows that one of its two constituent glycines occurs in the N-terminal hexapeptide Lys (Ala, Gly,Pro,Ser) Arg, and the other together with methionine and tryptophan in the C-terminal tryptic peptide (free of arginine and lysine), it is obvious that the •Gly•Try•Met• sequence occurs in the C-terminal part of the molecule. Should it be established by further work that it is the methionine of this tripeptide which is linked to the aspartyl-phenylalanine amide then cholecystokinin-pancreozymin and gastrin have not only the sequence •Asp•Phe•NH_2 but the pentapeptide Gly•Try•Met•Asp•Phe•NH_2 in common.

Experimental

3.6 mgs of the cholecystokinin-pancreozymin preparation was dissolved in 1 ml of 0.1 M HCl containing 3.6 mgs of CNBr and the solution kept at 21°C for 24 hours. It was then diluted with an equal volume of distilled water and lyophilized. The residue was taken up in 120 µls of distilled water and the solution applied for chromatography in aliquots of 3x1 µls to Whatman 42 paper. The spots were spaced on the starting line 0.5 cms apart. Descending chromatography was carried out for 20 hours at 21°C using the solvent 1-butanol-acetic acid-pyridine-water

(30 : 6 : 20 : 24) (Waley and Watson, 1954). Guide strips were stained with the ninhydrin reagent of Barrollier and coworkers (1957). In addition to poorly separated fragments in the region between the starting line and one third of the distance to the solvent front two well separated spots were evident, one of a clear yellow colour at a R_f value of 0.6, and one of the usual reddish colour at R_f 0.4. The material corresponding to the latter spot was eluted with one per cent acetic acid from the unstained part of the chromatogram and the eluate lyophilized. The residue was dissolved in 100 µls of distilled water. Acid hydrolysis (6 M HCl, 109°C, 24 h) of one tenth of the eluate and analysis of the hydrolysate in the Redfield (1953) system showed the presence of only two amino acids, aspartic acid and phenylalanine. Analysis of the hydrolysate, identically prepared, of another tenth of the eluate according to the Spackman, Stein and Moore technique (1958) showed 32 nanomoles of aspartic acid and 27 nanomoles of phenylalanine. Other amino acids occurred in trace quantities only. About 30 nanomoles of the dipeptide were subjected to high voltage paper electrophoresis (Locarte, 24 Emperors Gate, London, S.W.7, apparatus, Whatman 3 mm paper) at pH 6.4 (pyridine-acetic acid-water, by volume, 300 : 11.5 : 2700) and a potential drop of 50 v/cm for 40 minutes. It was found that the dipeptide behaved like a neutral substance. Consequently it could be assumed to be either asparaginylphenylalanine, phenylalanylasparagine, or aspartylphenylalanine amide. In order to determine which of these it was the dipeptide was subjected to one step Edman (1950) degradation according to Blombäck et al. (1966): 200 nanomoles in a conical centrifuge tube 1x7.5 cms, was dissolved in 200 µls of the pyridine-dimethylallylamine-trifluoroacetic acid buffer and incubated in an atmosphere of nitrogen at 40°C with 10 µls of phenylisothiocyanate. Another 5 µls of the latter was added after 1 hour and the incubation continued for a total of 2 hours. 200 µls of water was then added and the solution extracted 5 times with 1.5 mls of benzene. The benzene

washings were discarded and the aqueous layer lyophilized. The residue was taken up in 100 µls of anhydrous trifluoroacetic acid. After 20 minutes at 40°C the trifluoroacetic acid was removed *in vacuo* and the residue subjected to electrophoresis at pH 6.4 under the conditions described above. Staining of guide strips revealed only one ninhydrin positive component, which had migrated 15 cms towards the cathode. The material corresponding to the spot was eluted and an aliquot of it subjected to high voltage electrophoresis at pH 6.4 in parallel with synthetic phenylalanine amide (Yeda Research and Development Co. Ltd, Rehovoth, Israel). Both materials moved at the same rate. Another aliquot of the eluate was subjected to paper chromatography in the Waley and Watson (1954) system in parallel with the synthetic phenylalanine amide. Again both materials moved at the same rate.

Finally, ca. 30 nanomoles of the untreated dipeptide was chromatographed in parallel with synthetic aspartyl-phenylalanine amide (kindly given to us by Drs. M.A. Ondetti and J.T. Sheehan, The Squibb Institute for Medical Research, New Brunswick, N.J., U.S.A.). Both substances were indistinguishable by their mobilities.

The peptide from the cyanogen bromide degradation giving the yellow colour (typical of N-glycyl peptides) with the ninhydrin reagent used was eluted and an acid hydrolysate of it analysed in the Redfield (1953) system. Only glycine and homoserine were found. However, the peptide gave an intense reaction for tryptophan with p-dimethylaminobenzaldehyde (Smith, 1960).

Its N-terminal amino acid was determined by the "dansyl" technique of Gray and Hartley (Gray and Hartley, 1963; Smillie and Hartley, 1966). Hydrolysis of the dansyl peptide with 6 M HCl at 109°C was carried out for 4 hours only (David *et al.*, 1963). Only one dansyl amino acid, dansyl glycine, was found in the hydrolysate.

Paper electrophoresis at pH 6.4 separated the material into two

components, one neutral and one basic, both giving the same yellow colour with the ninhydrin reagent, a positive reaction for tryptophan, and an identical picture on acid hydrolysis. Consequently, the basic component could be assumed to be the lactone form of the tripeptide from which the neutral fraction had arisen on hydrolysis (Ambler, 1965). The neutral component was degraded with carboxypeptidase A (Worthington) under the conditions recommended by Canfield and Anfinsen (1963). Three amino acids were found on analysis of the enzymatic hydrolysate in the Redfield system. These were: glycine, homoserine, and tryptophan.

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