THE REACTION OF CHYMOTRYPSIN AND DIISOPROPYLPHOSPHOROFLUORIDATE

II. THE STRUCTURE OF TWO DP-SUBSTITUTED PEPTIDES FROM CHYMOTRYPSIN-DP*

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In the preceding paper[1] we reported the isolation of two DP-containing peptides from a proteolytic digest of chymotrypsin-DP. The quantitative amino acid analysis of one of these peptides (peptide B) revealed the presence of the following amino acid residues per bound DP-group: glycine (3), aspartic acid or asparagine (1), serine (1), proline (1), and leucine (1). The other peptide (peptide C) had an identical composition except for the absence of the leucine residue.

In this paper results pertaining to the amino acid sequence and the position of the DP-group of peptide B and C are reported.

EXPERIMENTAL

The peptides investigated were prepared according to the procedure described previously[1].

Reaction with 2,4-dinitrofluorobenzene (DNFB)

If not stated otherwise the reaction with DNFB was carried out as follows. The material (0.01–1 μmole of peptide) was dissolved in 0.1 ml of a 1% $NaHCO_3$ solution, and a solution of 3 mg of DNFB in 0.2 ml of ethanol was added. After standing for 2 h at 37°C, the ethanol was evaporated and the excess of DNFB removed by extraction with ether. The residual solution was acidified, and the extraction was repeated. Finally, the dinitrophenylated peptides (DNP-peptides) were recovered by an extraction with methyl acetate. Whenever the acidic ether extraction was omitted, the interfering dinitrophenol in the methyl acetate extract was sublimed off *in vacuo* (0.01 mm Hg) at room temperature.

Degradation with phenylisothiocyanate (PTC)

The stepwise degradation of peptide B from the amino end was performed according to the method of Edman as modified by FRAENKEL-CONRAT *et al.*[2]. After the reaction of the peptide with PTC, the terminal amino acid was released in the form of its phenylthiohydantoin (PTH-) by treatment with 3 *N* HCl at 37°C. The course of the PTH-formation was followed by observation of the development of the ultraviolet spectrum. The PTH-amino acids were extracted with ethyl acetate and identified by paper chromatography[3]. Moreover, the parent amino acids were identified by paper chromatography after hydrolysis of the PTH-amino acids in 6 *N* HCl at 150°C for 16 h in evacuated tubes.

Paper chromatography and high-voltage paper electrophoresis

These techniques were performed as detailed previously[1]. Solvents used for the amino acids were: phenol–ammonia; pyridine–water (4:1); butanol–acetic acid–water (BAW) (4:1:5). Solvents

* The abbrevations DP- for diisopropylphosphoryl-, and DP for diisopropylphosphate, will be used throughout.

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used for the PTH-amino acids were: heptane–pyridine (7:3) and the upper phase of heptane–butanol–formic acid (4:4:2). The spots were localized by ultraviolet absorption photography, rendering the usual impregnation of the paper (Whatman No. 1) with a starch solution unnecessary. The "Chromatolite" (Hanau) was used as a source of ultraviolet light and Gevaert document contact paper as the photo-sensitive material.

RESULTS

Amino-terminal analysis by the 2, 4-dinitrofluorobenzene (DNFB) method

It was shown in a previous paper[1] that peptide B consists of 3 glycyl, 1 aspartyl or asparaginyl, 1 seryl, 1 prolyl and 1 leucyl residues in addition to a diisopropylphosphoryl group; peptide C has the same composition except for the absence of leucine.

The investigation of the structure of these peptides was started with Sanger's DNFB method. Two μmoles of peptide B were dinitrophenylated and subjected to hydrolysis (6 *N* HCl, 50° C, 17 h). The DNP-derivatives produced by this partial hydrolysis were extracted with methyl acetate and separated by paper chromatography in BAW (Fig. 1). The spots a, b, and c, were eluted from the paper and chromatographed in diisopropyl ether–formic acid (3:2) (Fig. 1). Four DNP derivatives were thus obtained, of which the products a_1, b_1 and b_2, were analyzed. The compound located in spot b_2 was identified as DNP-glycine on chromatography in appropriate solvents[3], while no amino acids were released by a treatment with 6 *N* HCl at 105° C for 17 h. Therefore, glycine occupies the amino-terminal position of peptide B. Hydrolysis of the material eluted from spot b_1 yielded aspartic acid. In the hydrolysate of the eluate from the radioactive spot a_1 the presence of aspartic acid, glycine, and serine, was demonstrated.

From these results the amino-terminal sequence of peptide B could be established as: glycyl-aspartyl-(serine, glycine + ^{32}P). By means of analogous experiments it was shown that glycine is also the amino-terminal amino acid of peptide C.

Amino-terminal analysis by the Edman procedure

The structure of peptide B was further investigated according to the method of Edman as outlined by FRAENKEL-CONRAT *et al.*[2]. The product of the reaction of 4 μmoles of peptide B with phenyl isothiocyanate (PTC) was incubated with 3 *N* HCl at 40°C to achieve hydantoin formation (PTH). As judged from the optical density readings at 267 mμ, the maximal attainable amount of PTH was formed after an incubation of 2½ h. On extraction, the PTH-amino acid was recovered in 90% yield. It was identified as PTH-glycine by paper chromatography in heptane–butanol–formic acid and heptane–pyridine. Moreover, glycine was liberated from this compound on hydrolysis (5.7 *N* HCl for 16 h at 150°C).

Breaking of peptide bonds could easily occur during the prolonged HCl treatment necessary for the formation of PTH-glycine. To prevent faulty interpretation the residual peptide was, therefore, purified by high-voltage paper electrophoresis at pH 3.6 (2500 V for 2 h), before it was subjected to the next Edman procedure. The bulk of the radioactivity (82%) was found to be located in a single zone. The material of this zone was eluted and coupled with PTC. The second PTH-amino acid was obtained in a 92% yield after a treatment with 3 *N* HCl for 30 min at 40°C. This compound proved to be PTH-aspartic acid as demonstrated by comparison of its

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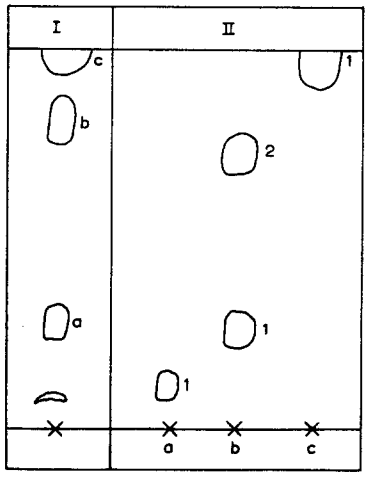

Fig. 1. Chromatogram in BAW (I) of DNP-derivatives extracted with methyl acetate from a partial hydrolysate of DNP-peptide B. The spots were eluted and rechromatographed in diisopropyl ether–formic acid (II).

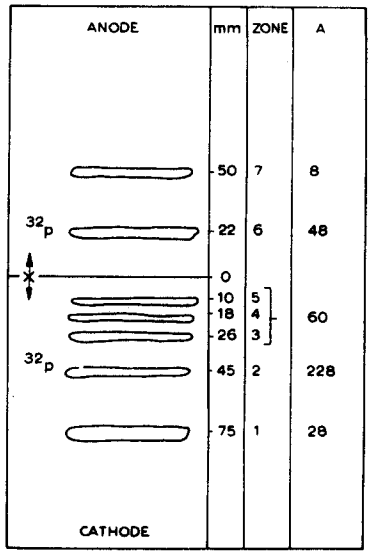

Fig. 2. High voltage electrophoresis at pH 3.6 (2500 V for 1 ½ h) of the solution obtained after applying twice the Edman procedure to peptide B. The light absorption (A) is recorded in arbitrary units.

R_F value on a paper chromatogram developed with heptane–butanol–formic acid with the R_F values of authentic samples of PTH-aspartic acid ($R_F = 0.57$) and PTH-asparagine ($R_F = 0.46$). In addition the parent amino acid was regenerated by hydrolysis and identified as aspartic acid.

The peptide resulting after the second PTH formation was purified and incubated with PTC. However no coupling was observed and a PTH-amino acid could not be obtained. During the incubation with PTC a large quantity of DP was liberated. Variation of the temperature and the pH did not lead to a satisfactory result.

In analogous experiments the amino terminal sequence of peptide C was shown to be glycyl-aspartyl. The common amino terminal sequence of glycyl-aspartyl, the related composition of both peptides and the fact that chymotrypsin-DP only contains one DP-group prove the carboxyl terminal position of leucine in peptide B.

Determination of the complete amino acid sequence

At this stage of the present investigations the structure of peptide B had been established as glycyl-aspartyl-(serine, glycine + ^{32}P) (glycine, proline)-leucine. The sequence of the amino acids between brackets could be deduced from analysis of the shortened radioactive peptide and other fragments obtained after two successive Edman degradations as follows. The Edman procedure was applied twice on 1 μmole of peptide B without intermediary purification. After the formation (3 *N* HCl at 40°C for 1 3/4 h) and extraction of the second PTH-amino acid the residual solution was fractionated by high voltage paper electrophoresis. The destructive action of the HCl is clearly demonstrated by the great number of products obtained (Fig. 2).

To gain some insight into the distribution of the material over the different zones,

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samples of their eluates were hydrolyzed (6 *N* HCl at 105°C for 17 h) and their amino acid content estimated by the ninhydrin method. In Fig. 2 the absorption is recorded in arbitrary units. It follows that the main product judged from the amino acid content was located in zone 2, while the zones 1 and 6 comprised fair and the other zones only minor quantities of material. Radioactivity measurements revealed that zone 2 contained 60% and zone 6 12% of the radioactivity originally applied to the paper.

From these data it seemed likely that the radioactive peptide, which was expected to result after two degradations, was located in zone 2 (peptide 2). Moreover, analysis of this peptide revealed the presence of serine (1), glycine (2), proline (1), and leucine (1), in addition to a DP-group. The radioactive peptide (peptide 6) located in zone 6 had an identical amino acid composition. However, the radioactive compound released by an alkaline treatment of peptide 6 (pH 11.0 for 8 min at 100°C) was identified as monoisopropylphosphate (MP) by comparing its R_F value in BAW with the R_F value of an authentic sample of MP ($R_F = 0.43$). The acidic nature of the MP-group in comparison with the DP-group gives a satisfactory explanation for the difference in electrophoretic behaviour of the peptides 2 and 6 (Fig. 2).

Of the non-radioactive products only the material eluted from zone 1 was completely identified. On chromatography in BAW two ninhydrin-positive spots 1A ($R_F = 0.80$) and 1B ($R_F = 0.69$) were obtained. Paper chromatography of hydrolyzed samples of the eluted material revealed the presence of proline and leucine in spot 1A, and of glycine, proline, and leucine, in spot 1B. The DNP-derivative obtained by dinitrophenylation of peptide 1A showed a maximal light absorption at 375 mμ in a 0.1 *N* HCL solution. This wavelength of maximal absorption is characteristic for DNP-prolyl derivatives and is indicative for the sequence prolyl-leucine. The DNP-derivative of 1B had a maximal absorption at 350 mμ. The DNP-amino acid was isolated and identified as DNP-glycine by paper chromatography. Total hydrolysis of the DNP-derivative of 1B yielded glycine, proline and leucine in nearly equimolar ratios. (No glycine is regenerated from DNP-glycine under these circumstances.)

From these data and the known carboxyl-terminal position of leucine the following amino acid sequence was deduced: glycyl-glycyl-prolyl-leucine. It seems justified in view of these results to conclude that the amino acid sequence of peptide 2 should be seryl-glycyl-glycyl-prolyl-leucine*. Several efforts were made to confirm the N-terminal position of the serine residue in peptide 2 in a more direct way.

As mentioned above, efforts directed to isolate the PTH-derivative of peptide 2 had been unsuccessful. The reaction with DNFB under the normal conditions (10 mg DNFB/ml; 3 h at 37°C) proceeded rather slowly while a large quantity of DP was released. Only 65% of the radioactivity appeared to be extractable with methylacetate while 75% of the extracted radioactivity was shown to be present as DP. This experiment was repeated with a more concentrated solution of DNFB and a shorter reaction time (40 mg DNFB/ml; 1 h at 37°C). 70% of the radioactivity was then extractable with methylacetate. The extract was analyzed by paper chromatography in BAW. After resolution, 23% of the radioactivity appeared to be present as DP; the remainder coincided with the yellow spot of a DNP-derivative ($R_F = 1.0$). The eluted DNP-derivative was hydrolyzed (2 *N* HCl at 100°C for 2 h), and the

* In view of the difficulty of quantitative interpretation of the exact number of glycyl residues (in the presence of a relatively large number of such residues) it should be noted that a sequence -seryl-glycyl-glycyl-glycyl-prolyl- is not out of the question at this stage.

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hydrolysate was subjected to paper chromatography in BAW, diisopropyl ether–formic acid (3:1), and 1.5 *M* phosphate buffer (pH 6), adding synthetic N-DNP-O-phosphoryl-serine* as a marker substance. Most of the radioactivity coincided with the yellow colour of the marker substance revealing the presence of radioactive N-DNP-O-phosphoryl serine in the hydrolysate.

From the results presented, the sequence of the amino acids of peptide B can be established unequivocally as: glycyl-aspartyl-seryl-glycyl-glycyl-prolyl-leucine.

Position of the DP-group

The results presented so far have furnished some indications about the position of the DP-group: a comparison of the radioactive peptide 2 with the non-radioactive fragment, glycyl-glycyl-prolyl-leucine, suggested a relation between serine and the DP-group. Also, the anomalous behaviour of the peptide with the amino terminal serine (peptide 2) towards the reaction with PTC and DNFB points toward such a relation. A strong support for a serine–DP relation was the isolation of N-DNP-O-phosphoryl serine. Additional evidence for a substitution of the DP-group to the serine residue was obtained from other experiments.

After acid hydrolysis (2 *N* HCl at 100°C for 5 h) of peptide B, O-serine phosphate could be demonstrated in the hydrolysate by paper chromatography in isobutyric acid–0.5 *N* ammonia (10:6) (R_F = 0.32) and BAW (R_F = 0.07). The behaviour of peptide B in alkaline solutions was very remarkable. As mentioned before, the bound DP-group was liberated by heating the peptide at 100°C for 4 min in 0.01 *N* NaOH solution. After total hydrolysis (5.7 *N* HCl at 105°C for 16 h) of the solution thus obtained all expected amino acids were detected with the exception of serine. Evidently, the elimination of the DP-group is accompanied by a destruction of serine. Since it was expected[8] that the release of the DP-group would involve the dehydration of the seryl residue leading to a dehydroalanyl derivative it was attempted to demonstrate the occurrence of such an intermediary.

A portion of alkaline-treated peptide B was purified by high-voltage electrophoresis at pH 3.6 (2500 V for 1 h). The bulk of the ninhydrin-positive material was ^{32}P-free and had travelled 10 mm towards the cathode; it was eluted and hydrogenated for 16 h in pyridine–acetate (pH 5) solution with hydrogen and using charcoal–palladium as a catalyst**. The recovered peptide was submitted to total hydrolysis. In the hydrolysate, the presence of each of the amino acids of peptide B could be demonstrated but instead of serine a nearly stoichiometrical amount of alanine was found.

It was peculiar that alanine rather than serine also resulted from straightforward hydrolysis of peptide B in 0.3 *M* barium hydroxide (105°C for 16 h). By means of quantitative paper chromatography the molar ratios of aspartic acid, serine, glycine, proline, leucine, and alanine, in the hydrolysate could be established as: 0.9:0.1:2.2:0.9:1.0:0.7. It may be concluded from these ratios that the production of alanine was accompanied by the destruction of the serine residue and one of the glycine residues. In the discussion it will be shown that these facts are consistent with an attachment of the DP-group to the hydroxyl of serine.

* This compound was prepared from O-serine phosphate and DNFB.

** The use of pyridine to prevent adsorption was inspired by the methylation procedure as employed by R. G. SHEPHERD *et al. J. Am. Chem. Soc.*, 87 (1956) 5058.

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DISCUSSION

In a previous paper[1] it was shown that there is reason to believe that the DP-substituted peptides (peptides B and C) obtained from a proteolytic digest of chymotrypsin-DP correspond to that part of the peptide chain of chymotrypsin-DP to which the DP-group is substituted. Therefore, elucidation of the structure of these DP-substituted peptides should give valuable information on the DFP binding site of chymotrypsin. Previously, full evidence has given for the purity of peptide B and peptide C. In this paper it has been shown that the amino-acid sequence of peptide B is as follows: glycyl-aspartyl-seryl-glycyl-glycyl-prolyl-leucine. Peptide C has an identical structure except for the absence of the terminal leucine residue.

The sequence, glycyl-aspartyl-seryl-glycyl, confirms the findings of other investigators; however a discrepancy remains with respect to the remaining amino acids. SCHAFFER *et al.*[4], who did pioneer work on the DFP-binding site of chymotrypsin, isolated a peptide, glycyl-aspartyl-phosphoseryl-glycine, from a partial acid hydrolysate of chymotrypsin-DP. However, they claimed that this sequence should be extended to: glycyl-aspartyl-phosphoseryl-glycyl-glutamic acid[4]. Besides, these investigators were able to isolate a peptide from chymotrypsin inhibited with Sarin. This peptide contained the following amino acids in unknown relative quantities: glycine, aspartic acid, methylphosphonylserine, glutamic acid, alanine, and valine. The N-terminal sequence was glycyl-aspartyl, while alanine and valine were released with carboxypeptidase. Though a comparison of this peptide and peptide B is difficult for lack of quantitative data, the absence of proline and leucine in addition to the carboxyl-terminal position of alanine or valine are not consistent with the proposed structure of peptide B.

After proteolysis with papain, SCHAFFER *et al.*[5] obtained a peptide composed of glycine, aspartic acid, methylphosphonylserine, glutamic acid, alanine, valine, histidine, proline, leucine, cystine, and threonine. This composition is not inconsistent with our results.

TURBA *et al.*[6] partially confirmed the results of SCHAFFER *et al.*[4] and ourselves by the isolation of a phosphorus-containing peptide aspartyl-seryl-glycine. However, the tentatively proposed structure of another phosphorus-containing peptide aspartyl-seryl-glycyl-glutamyl-alanine is not in accordance with our results.

Some difficulties were encountered in the establishment of the position of the DP-group. The fact that the radioactivity of the peptides coincides with the presence of serine suggests that the DP-group is attached to this residue. The anomalous behaviour with regard to the reaction with PTC and DNFB of the serine N-terminal peptide 2, involving the release of DP, indicates the same. The formation of O-serine phosphate on acid hydrolysis of peptide B, and the isolation of DNP-serine phosphate from peptide 2 seems to complete the evidence that the DP-group is attached to the hydroxyl of serine. However, it is not justified to conclude from these findings that the DP-group originally occupies this position. A migration of the DP-group from the NH_2 to the OH of serine as a result of the acidic treatment involved in the Edman procedure and the straightforward hydrolysis of peptide B cannot be excluded if the possibility of the presence of an ester linkage in the "peptide" B is taken into account. Two structures are then possible:

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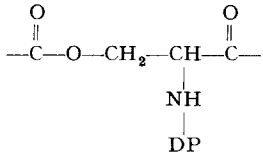

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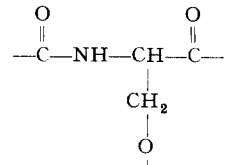

II

However, the behaviour of peptide B on the alkaline treatment provides evidence for structure II. RILEY *et al.*[8] report that O-diphenyl phosphoryl serine methyl ester yields, under similar conditions, diphenyl hydrogen phosphate and a dehydroalanyl derivative that ultimately yields pyruvic acid. It seems likely that the reaction mechanism involved in the destruction of the serine residue by successive alkaline and acidic treatment of peptide B is analogous to that observed by these authors.

The absence of serine in the acid hydrolysate of the alkaline-treated peptide B and the appearance of alanine after hydrogenation is accounted for if the eliminaion of the DP-group is accompanied by the formation of a dehydroalanyl residue in the peptide chain. Also, the results obtained by hydrolysis of peptide B with barium hydroxide favour this conception. The destruction of serine in this case involves the formation of alanine while the glycine content is decreased. SHAN CHENG *et al.*[9] have shown that pyruvoylglycine is converted into alanine by a successive treatment with alkali and acid. The formation of alanine on alkaline hydrolysis of peptide B suggests the occurrence of pyruvoylglycine during the hydrolysis. This intermediary could easily result from a dehydroalanyl peptide. A satisfactory explanation for the formation of a dehydroalanyl-derivative can be offered only if structure II is accepted.

In the preceding paper it was argued that peptide B corresponds to that fragment of chymotrypsin-DP that carries the DP-group. It does not follow that the serine residue of peptide B is involved in the primary reaction of DFP with the enzyme. The increasing resistance to reactivation by nucleophilic reagents on prolonged incubation of esterases-DP ("ageing") means undoubtedly that changes occur after the initial reaction of DFP with the enzyme[11]. It has been suggested[7,10] that these changes are due to a migration of the DP-group from the initial point of attack of DFP on the enzyme to the hydroxyl group of a serine residue. A number of investigators[12–16] presented evidence that a histidine residue may be involved in the enzymic and DFP-binding properties of chymotrypsin. It is attractive to assume that this residue is involved in the primary attack of DFP on chymotrypsin and that on ageing, transfer of the DP-group to the serine residue occurs. The absence of histidine in peptide B should not be regarded as strong evidence against this assumption. The required adjacent position of serine and histidine in the enzyme molecule could be satisfied by the sterical configuration of the enzyme. However, the phenomenon of ageing does not necessarily imply a migration of the DP-group. It has been found that horse liver aliesterase-DP is not subject to ageing even after prolonged incubation[17]. However, denaturation caused a stabilisation of the DP-group, which could no longer be removed by nucleophilic reagents. It seems likely that a larger part of the protein molecule is involved in the reactivation process and that the necessary cooperation of essential groups is no longer possible after denaturation.

Whatever reactions may occur after the initial reaction of DFP with the enzyme,

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the conclusion that the serine residue plays an important role in the inactivation process seems difficult to avoid. The isolation of O-serine phosphate from DFP-inhibited chymotrypsin[18,19], horse liver aliesterase[17], serum pseudocholinesterase[20], electric eel true cholinesterase[21], bovine true cholinesterase[20], and aliesterase (stroma)[20], and the isolation of closely related DP-substituted peptides[22,23] from trypsin, chymotrypsin and horse liver aliesterase, which peptides again produce O-serine phosphate on acid hydrolysis, suggest that these esterases have a definite serine-containing structure in common. It seems justified to correlate this serine-containing structure with functional properties also common to these proteins, namely, their ability to combine with DFP and to split esters.

SUMMARY

1. The amino acid sequence of two diisopropylphosphoryl-substituted peptides obtained from α-chymotrypsin-DP by enzymic hydrolysis was established as: glycyl-aspartyl-seryl-glycyl-glycyl-prolyl-leucine and glycyl-aspartyl-seryl-glycyl-glycyl-proline, respectively.

2. The experimental results strongly suggest that the diisopropylphosphoryl group is attached to the hydroxyl group of the seryl residue.

3. The significance of the investigated peptides in respect to the DFP-binding and ester-splitting ability of α-chymotrypsin is discussed.

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