*THE SEQUENCE AROUND THE ACTIVE-CENTER TYROSYL RESIDUE OF BOVINE PANCREATIC CARBOXYPEPTIDASE A**

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We have previously reported the isolation of peptides containing the tyrosyl residue from the active center of bovine carboxypeptidase A (Cpase A).[1] Based on that work we now report that this tyrosyl residue is in the sequence isoleucyl-tyrosyl-glutam(in)yl-alanyl.

The isolation of this peptide depended on a pair-labeled iodination procedure in which one portion of Cpase A was iodinated with I^{125}-labeled hypoiodite and a second portion was iodinated with I^{131}-labeled hypoiodite in the presence of β-phenylpropionate to protect the site from iodination. The preparations were mixed, digested by elastase, and the iodinated peptides separated. Analysis of the ratio of I^{125}/I^{131} for isolated peptides revealed that one major iodinated tyrosyl peptide had a ratio higher than the others, showing that this tyrosyl residue is in the active center since its exposure to iodination was blocked by the inhibitor.

This tyrosyl residue appears to be the one studied by others. Riordan *et al.*[2] have recently reported that under controlled conditions tetranitromethane nitrates a single tyrosyl residue in Cpase A. This tyrosyl residue also appears to be the one in the site, since they found that the presence of the inhibitor β-phenylpropionate protects the site against this reagent and essentially no nitration occurs. Furthermore, they found that the protected Cpase A retains native enzyme activity while the unprotected mononitrated Cpase A has only 10 per cent of the native peptidase activity and has 170 per cent of the native esterase activity. Similar behavior of the enzyme activity of Cpase A has been previously reported by Vallee *et al.*[3] upon chemical alteration of the tyrosyl residues by iodination and acetylation in the presence and absence of the inhibitor.

Materials and Methods.—Bovine pancreatic carboxypeptidase A (Cpase A) (diisopropylfluorophosphate-treated) and elastase (electrophoretically purified) were obtained from the Worthington Biochemical Corp., Freehold, N. J. Pronase was obtained from Biddle Sawyer Co., New York, N.Y.

Concentrations of iodinated Cpase A were based on Nesslerization following digestion.

Iodinations were carried out in a manner similar to that previously reported,[1] with the following changes: The iodinations were carried out at pH 7.5 using a Tris-NaCl buffer; the β-phenylpropionate concentration was 0.03 *M;* and the Cpase A suspension was dissolved directly in Tris-NaCl buffer to a concentration of 8 mg/ml without the aid of 0.1 *M* NaOH. Tris-NaCl buffer was prepared by adding 8.4 ml of concentrated HCl and 8.7 gm of NaCl to about 60 ml of water and bringing this solution to pH 7.5 by the addition of solid Tris (about 14 gm). The volume was then brought to 100 ml with water.

Following the iodinations, each reaction mixture was dialyzed in the following order: against 8 liters of cold 1 *M* saline for 8 and then for 16 hr; against 0.05 *N* NaOH for 18 hr at room temperature;[4] twice against 8 liters of cold water; and finally against 8 liters of cold 0.01 *M* formic acid. The contents were removed from the bags and the protein nitrogen and radioactivity were determined.

For enzymatic digestion of the pair-iodinated Cpase, suitable portions of the two preparations

were mixed and lyophilized. Digestion of iodinated Cpase A by pepsin was carried out as previously described.[1] For elastase digestions, 1 ml of 0.1 *M* ammonium bicarbonate containing 0.25 mg of elastase per ml was used for each 10 mg of lyophilized, iodinated Cpase A. A drop of toluene was added and the digestion was performed for 18 hr at 37°.

High-voltage paper electrophoresis (HVPE), chromatography, radioautography, and determination of I^{125} and I^{131} in the labeled iodinated peptides were carried out as previously described.[1]

Partial purification of the high-ratio iodinated peptide from the elastase digest was carried out by means of column chromatography. A jacketed 0.9 × 105-cm column of Dowex 50 (200–400 mesh) was equilibrated with starting buffer at 35°. The digest, containing up to 45 mg in a 5-ml volume, was brought to about pH 2. It was applied to the column and subjected to gradient elution. The gradient was derived from two connected cylinders, one containing 450 ml of starting buffer being continuously stirred and the other containing 450 ml of final buffer. The eluting solution was pumped through the column at 20 ml/hr and 5-ml fractions were taken. The starting buffer of pH 3.2 contained 6.7 ml of 85% H_3PO_4, 6 ml of concentrated NH_4OH, and 300 ml of ethanol in a total volume of 1 liter. The final buffer of pH 9.1 contained the same volume of H_3PO_4 and ethanol but contained 18 ml of concentrated NH_4OH.

The determination of the amino acid sequence of the peptide was patterned after the procedure of Gray and Hartley.[5] The N-terminal residue was labeled with 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) and the DNS-peptides were purified by paper electrophoresis at pH 6.5 according to Gray and Hartley.[5] Following elution and acid hydrolysis, the DNS-amino acid was identified by thin-layer chromatography using Eastman type K301R plates and solvent systems of Deyl and Rosmus.[6] The Edman degradations were done according to the method of Konigsberg and Hill.[7] The procedure was scaled down and was followed to include the cyclization step and the purification of the degraded peptide by benzene extraction. Their subsequent purification step on Dowex 50 was not included.

Results and Discussion.—Enzymatic digestion of pair-labeled mixtures: Elastase digestion resulted in a product which, upon HVPE and chromatography, gave good resolution of the peptides with little radioactivity remaining at the origin. A radioautograph is reproduced in Figure 1. One peptide (no. 2) showed a higher ratio than the others and contained a major amount of radioactivity. The ratio I^{125}/I^{131} and the counts per minute of I^{125} for each spot are shown on Figure 2.

When pepsin was used to digest another portion of this pair-iodinated mixture, the pattern of the radioiodinated peptides was, of course, different from that of elastase. The pattern for the peptic digest was as reported previously[1] and one spot (no. 17 in that report) again showed a high ratio and contained a major amount of the total I^{125}.

Although the positions of the high-ratio spots were different for the elastase and peptic digests, indicating that the peptides were different, the ratio I^{125}/I^{131} and the I^{125} counts per minute in the peptide from each digest were in close agreement. Thus, the same iodinated tyrosyl residue was involved in both. This close agreement in the ratio and amount of the two peptides suggests that although elastase and pepsin cleave the active center sequence containing this iodinated tyrosyl residue at different bonds, each releases all of this residue as a single peptide, specific for the particular enzyme.

In tryptic digests, the iodinated peptides were not well resolved and considerable radioactivity remained at the origin of the chromatogram. No peptide with a comparably high I^{125}/I^{131} ratio was found.

Isolation of high-ratio peptide on a preparative scale: Elastase was used for further work since it gave a good yield of the iodinated peptide containing the tyrosyl residue from the active center of Cpase A and this peptide was better separated from other peptides than in the case of peptic peptides. The high-ratio peptide

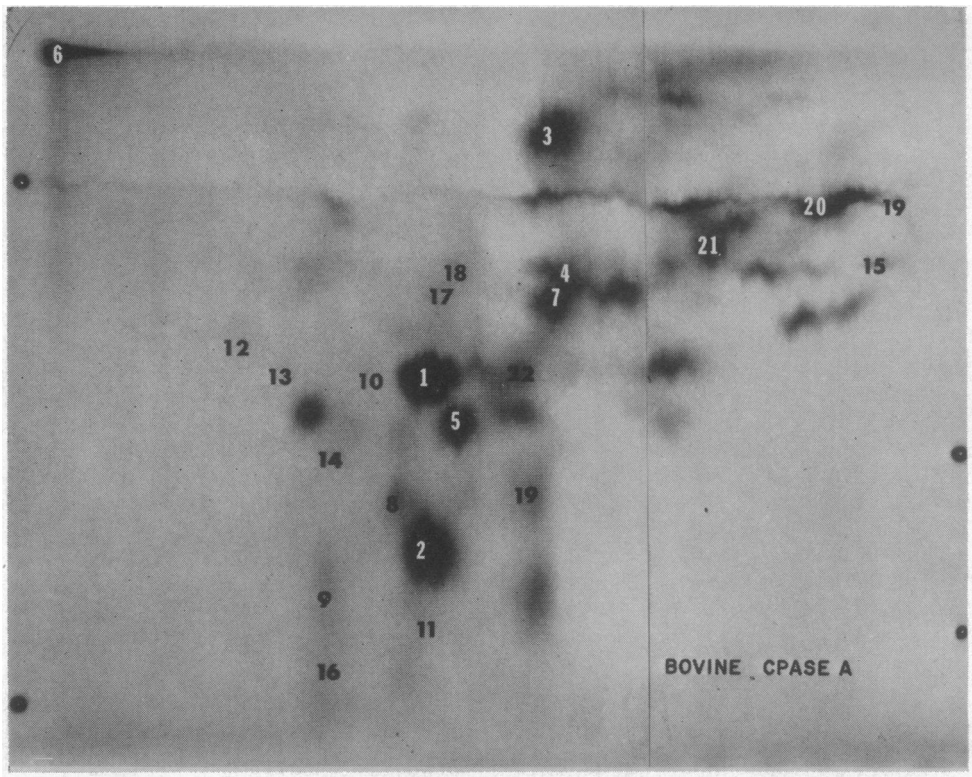

FIG. 1.—Radioautograph of the pattern of peptides separated from an elastase digest of iodinated carboxypeptidase by chromatography and high-voltage paper electrophoresis.

for sequence determination was partially purified by column chromatography on Dowex 50. Preliminary experiments showed that iodinated peptides bind strongly to Dowex 50 and are not eluted under conditions which ordinarily elute uniodinated peptides. The column chromatography system described in *Materials and Methods* was therefore developed and the high-ratio peptide was isolated from the elastase digest of pair-iodinated Cpase A. The pair-iodinated mixture was prepared with a larger amount of Cpase A iodinated in the absence of inhibitor and a smaller amount of Cpase A iodinated in the presence of inhibitor, since the labeled peptide comes primarily from the former preparation. The iodinations were carried out accordingly.

Five ml of iodinating solution containing 34.5 μmoles of ICl and 550 μc of I^{125} was added to 5.4 ml of Cpase A solution (1.25 μmoles) in the unprotected preparation while in the protected preparation 0.84 ml of a solution containing 3.45 μmoles of ICl and 440 μc of I^{131} was added to 0.54 ml of the Cpase A solution (0.125 μmole) containing 0.03 *M* β-phenylpropionate.

Following dialysis, both the protected and unprotected preparations were found to have an average of 16.5 atoms of iodine incorporated per molecule of Cpase A.

The two preparations were mixed and digested with elastase. The digest was lyophilized, taken up in 5 ml of starting buffer, and placed on a Dowex-50 column and the gradient was started. Five-ml fractions were collected. The elution

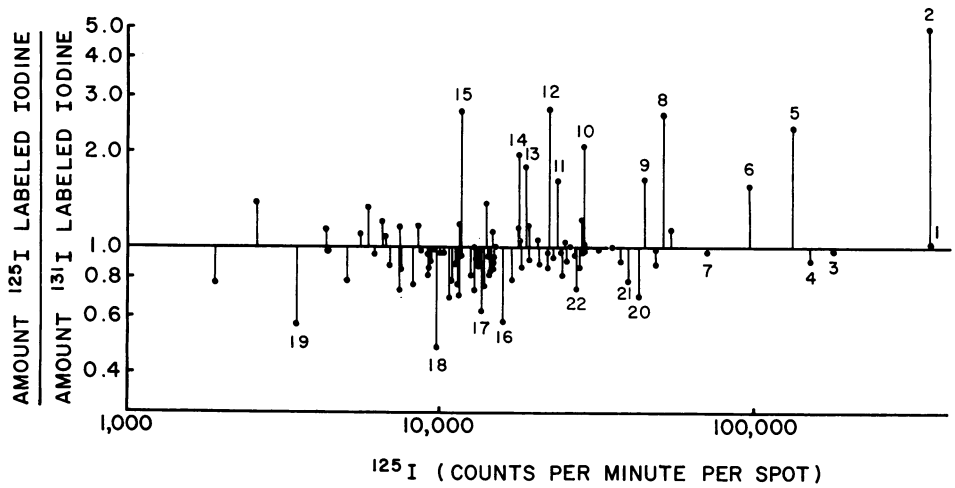

FIG. 2.—Radioactivity present in the iodine-containing peptides from an elastase digest of pair-labeled carboxypeptidase. The ratio of I^{125}/I^{131}-labeled iodine for each spot obtained is shown. The values are arranged in the order of increasing I^{125} in the spots. Both axes are logarithmic.

pattern is shown in Figure 3. This gradient, in preliminary experiments, had been shown to move peptide no. 2, isolated from paper (Fig. 1), in a satisfactory manner. When the whole digest was put through the column, a peak showing a high ratio (Fig. 3) was eluted around fraction no. 87. This position corresponds to the position on the gradient taken by peptide no. 2 isolated from the paper and determined in preliminary experiments. Furthermore, nearly all of the material in this peak of

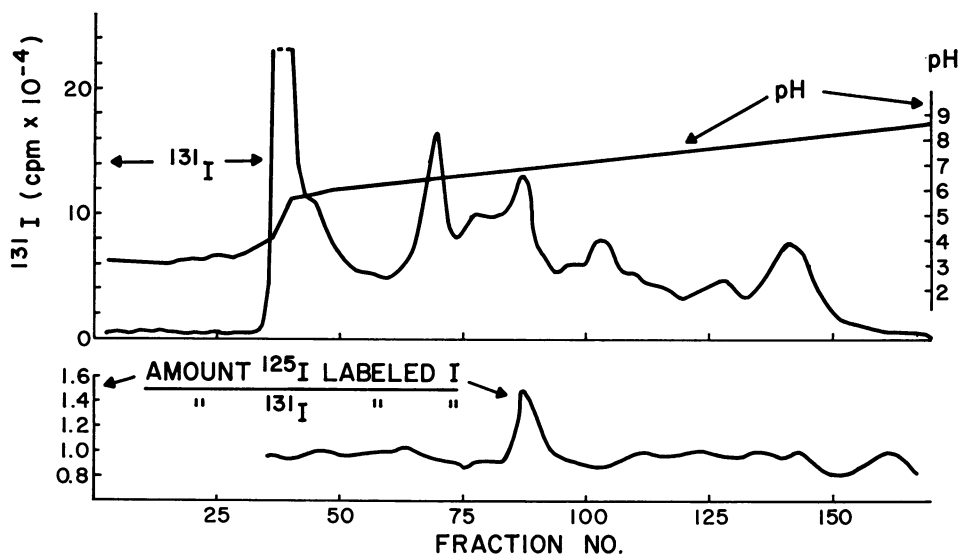

FIG. 3.—Elution of the iodinated peptides in an elastase digest of carboxypeptidase A from Dowex-50 column by gradient shown. The ratio of the amount of I^{125}-labeled peptide of I^{131}-labeled peptide in the eluate is shown at the bottom.

the eluate behaved the same on subsequent HVPE and paper chromatography as peptide no. 2. These results confirm the identity of the column-isolated peptide and peptide no. 2 from the paper.

The fractions composing the high-ratio peak in Figure 3 were pooled and the pool was brought to pH 2 with hydrochloric acid. The iodinated peptides in the pool were adsorbed on a 12.5 × 1.25-cm column of Dowex 50 equilibrated with 1 *M* acetic acid, and the phosphate and chloride were removed by washing the column with 25 ml of 1 *M* acetic acid followed by 25 ml of water. The adsorbed peptides were then eluted with 1 *M* ammonium hydroxide. The recovery of radioactivity (and iodinated peptides) that had been in the pool was essentially 100 per cent and was in a volume of 3–4 ml. This eluate was lyophilized.

The high-ratio iodinated peptide in this eluate was further purified by HVPE and chromatography using the same system as was used to obtain the pattern shown in Figure 1. The column fractionation reduced the total amount of material to such an extent that it could all be run on a single paper. A radioautograph was prepared by a two-hour exposure of the film and nearly all of the radioactivity was found in the position of peptide no. 2; several smaller spots, well separated from the major spot, were also found. There were no uniodinated, ninhydrin-positive peptides in the vicinity of this spot; such peptides move faster, on the average, than iodinated peptides in this electrophoretic system. The material in the major spot was eluted from the paper with 1 *M* ammonium hydroxide and the eluate was lyophilized. The yield of peptide-coupled iodine was 0.4 μmole. A small portion of the iodinated peptide was digested with pronase and pancreatin and the digest, to which some known I^{131}-labeled monoiodotyrosine (MIT), diiodotyrosine (DIT), monoiodohistidine (MIH), and diiodohistidine (DIH) had been added,[8] was subjected to HVPE.[9] The I^{125}-labeled material in the digest migrated to the position of the I^{131}-labeled DIT, indicating that the iodinated residue in the peptide was DIT. Thus the yield of peptide in this particular run was 0.2 μmole assuming only one diiodotyrosyl residue in the peptide.

The amino acid composition of the eluted peptide was determined on 0.05 μmole. The peptide was hydrolyzed with constant boiling HCl in a sealed tube at 107° for 22 hours. The hydrolyzate was analyzed on a Technicon automatic analyzer using a 0.3 × 125 cm Dowex-50 column and a gradient similar to that of Piez and Morris.[10] The amino acids from the peptide were found to be alanine, isoleucine, tyrosine,[11] and glutamic acid in essentially an equimolar ratio, 1.00:0.94:0.87:1.18. Glycine was present at 0.1 mole ratio; leucine at 0.03; aspartic, threonine, and serine at less than 0.02 each. Other amino acids were not detected.

Sequence of the amino acids in the peptide: The sequence was determined starting with approximately 0.2 μmole of the peptide. In the first stage, 0.025 μmole of the peptide was labeled with DNS-Cl. Flat-bed electrophoresis of the product at pH 6.5 gave a yellow fluorescent band with a mobility of 0.91 relative to the sulfonic acid.[5] The DNS-peptide in the band was eluted and hydrolyzed. The DNS-amino acid in the hydrolyzate was identified as DNS-isoleucine by thin-layer chromatography.

In the second stage, the remaining untreated peptide (0.175 μmole) was subjected to Edman degradation and a portion equivalent to 0.044 μmole was treated with DNS-Cl. When this material was subjected to electrophoresis at pH 6.5,

only the blue fluorescence of the acid and the yellow fluorescence of the amide appeared with no evidence of a DNS-peptide. This behavior, together with the knowledge of the composition of the peptide, showed that the N-terminal residue of this peptide is DIT. Thus, the second residue of the active-center peptide as isolated is diiodotyrosine. In previous runs we had encountered this situation at this stage and investigation of known N-terminal DIT-peptides showed that the DNS-derivatives of such peptides are apparently not formed under these conditions. However, we did find that an N-terminal DIT residue is released in the Edman degradation as carried out here and the resulting new N-terminal residue reacts with DNS-Cl and can be determined without difficulty. Therefore, the remaining DIT-N-terminal peptide (0.130 μmole) was degraded and one half of the DIT-free peptide was treated with DNS-Cl. The resulting DNS-peptide had a mobility of 0.70 relative to the DNS-acid at pH 6.5. Upon elution, hydrolysis, and thin-layer chromatography, the DNS-amino acid was found to be DNS-glutamic acid. The electrophoretic behavior of the DNS-peptide suggests glutamine, rather than glutamic acid, as the residue in the peptide, however.

The remaining DIT-free peptide (0.065 μmole and presumably glutam(in)yl-alanine) was degraded. The last residue was treated with DNS-Cl and its behavior on electrophoresis at pH 6.5 and in thin-layer chromatography agreed with that of known DNS-alanine.

Thus, the active center of bovine pancreatic Cpase A contains the sequence isoleucyl-tyrosyl-glutam(in)yl-alanyl.

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[1] Roholt, O. A., G. Radzimski, and D. Pressman, these PROCEEDINGS, **53**, 847 (1965).

[2] Riordan, J. F., M. Sokolovsky, and B. L. Vallee, *Biochemistry*, **6**, 358 (1967).

[3] Vallee, B. L., J. F. Riordan, and J. E. Coleman, these PROCEEDINGS, **49**, 109 (1963).

[4] This dialysis against 0.05 *N* NaOH makes the Cpase more readily soluble in 0.1 *M* ammonium bicarbonate, thus permitting better hydrolysis by the elastase. Peptic digests of this material show the same peptide pattern as peptic peptides of iodinated Cpase not exposed to the alkaline treatment.

[5] Gray, W. R., and B. S. Hartley, *Biochem. J.*, **89**, 379 (1963).

[6] Deyl, Z., and J. Rosmus, *J. Chromatog.*, **20**, 514 (1965).

[7] Konigsberg, W., and R. J. Hill, *J. Biol. Chem.*, **237**, 2547 (1962).

[8] The I^{131} of the iodinated peptide had decayed to a negligible value.

[9] Dube, S. K., O. A. Roholt, and D. Pressman, *J. Biol. Chem.*, **239**, 1809 (1964).

[10] Piez, K. A., and L. Morris, *Anal. Biochem.*, **1**, 187 (1960).

[11] This amount of tyrosine is essentially equivalent to the amount of diiodotyrosine in the peptide. An equimolar mixture containing 0.05 μmole each of isoleucine, diiodotyrosine, glutamic acid, and alanine in 6 *N* HCl was heated at 107° for 20 hr in a sealed tube and then analyzed on the same column. The yields in μmoles were: isoleucine, 0.041; tyrosine, 0.045; glutamic acid, 0.043; and alanine, 0.048.