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LIMITED PROTEOLYSIS PATTERNS OF THE B CHAIN OF INSULIN

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SUMMARY

The oxidized B chain of insulin was used as a simple model for further consideration of limited proteolysis with low substrate:enzyme ratios. With low B chain:trypsin ratios, the ordinarily slower cleavage rate of the -Lys_{29}-Ala_{30} bond essentially equaled the cleavage saturation rate of the -Arg_{22}-Gly_{23} bond. This led to the disappearance of octapeptide which ordinarily forms most rapidly. Heptapeptide and alanine, formed mainly by cleavage of the octapeptide, decreased somewhat at high enzyme relative levels. Trypsin added to B chain formed a single chromatographic peak.

INTRODUCTION

This study was initiated as an extension of work with a navy bean component (1) that varies in protease specific inhibiting capacity according to the ease with which various substrates are cleaved (2, 3). The oxidized B chain of insulin was chosen as a substrate model for further examination of differential inhibition since trypsin hydrolyzes only two bonds of the B chain and cleaves the -Arg_{22}-Gly_{23} bond (forming an octapeptide and B chain desoctapeptide) more rapidly than the -Lys_{29}-Ala_{30} bond of the octapeptide is cleaved (4-6). Alanine is the C-terminal residue. Although the inhibitor magnifies the difference in these cleavage rates, it retards both. As a first step, additional details were sought regarding cleavage patterns with varying amounts of uninhibited trypsin. The results obtained with this model are reported here. Attention was given to low substrate:enzyme ratios since they are of interest under subcellular conditions.

Abbreviations:NPGB, p-nitrophenyl p'-guanidinobenzoate; TPCK-trypsin, trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone; S:E, substrate: active enzyme molar ratio.

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MATERIALS AND METHODS

Reaction products were quantitated with an amino acid analyzer essentially according to the procedure of Wang and Carpenter (5). The resin of the chromatographic column was Aminex A-5 (Bio-Rad Laboratories). Pyridine for the preparation of pyridine acetate buffers was purified by glass distillation of analytical grade pyridine after adding 3 g of ninhydrin per liter. The ninhydrin solution containing dimethylsulfoxide was prepared according to Moore (7) with reagents obtained from the Pierce Chemical Company. Trypsin treated with L-(tosylamide 2-phenyl) ethyl chloromethyl ketone to inhibit any residual chymotryptic activity (TPCK-trypsin) was purchased from the Worthington Biochemical Corporation. Bovine oxidized B chain of insulin was purchased from Boehringer Mannheim Biochemicals. The molarity of active TPCK-trypsin based on molecular weight of 23,800 (8) was determined by the p-nitrophenyl, p'-guanidinobenzoate (NPGB) titration method of Chase and Shaw (9). The concentration of the enzyme is expressed (except as noted) as the active form which was 63.5% of the total trypsin.

The concentration of insulin B chain was 0.5 micromoles per ml of reaction solution in all cleavage experiments. The substrate was dissolved in 0.1 M Tris buffer, 0.01 M $CaCl_2$, pH 8. TPCK-trypsin was dissolved in 1 mM HCl, 0.01 M $CaCl_2$ in an ice bath. Aliquots (200 to 500 µl) were promptly transferred to 12 x 100 mm tubes and each immediately frozen in an alcohol-dry ice bath. To one of these frozen preparations an appropriate volume of diluting 1 mM HCl was added. As soon as the enzyme solution melted, the diluted trypsin was mixed and 500 µl were placed in a 12 x 100 mm tube, put in a 30° bath, and rocked continuously. Two minutes later 500 µl of buffered substrate were added from a microsyringe. One minute later 1 ml of 0.2 N HCl was added to stop the reaction and the sample was frozen in an alcohol-dry ice bath. It was kept frozen until analyzed. Each trypsin aliquot was treated in this manner. One ml of the acidified solution was used for analysis of the cleavage pattern with the amino acid analyzer. The chromatographic peaks were integrated by multiplying the height of a peak by the width at half the height (10).

RESULTS

The peptide and alanine pattern resulting from the limited proteolysis of the oxidized B chain of insulin by TPCK-trypsin in the substrate to active enzyme molar ratio (S:E) range of 0.5 to 600 is shown in Figs. 1 and 2. With S:E = 600, there was enough enzyme to produce in 1 min (at 30° and pH 8) an appreciable amount of octapeptide from the cleavage of the more rapidly reacting $-Arg_{22}-Gly_{23}$ bond, but only trace amounts of the heptapeptide and alanine formed as the result of the cleavage of the slower reacting $-Lys_{29}-Ala_{30}$ bond of the octapeptide (Fig. 2). The effects of increasing the enzyme are shown in Figs. 1 and 2. The yield of octapeptide increased and then decreased approaching zero at S:E = 10. The heptapeptide and alanine reached maxima at somewhat lower S:E ratios and then decreased moderately.

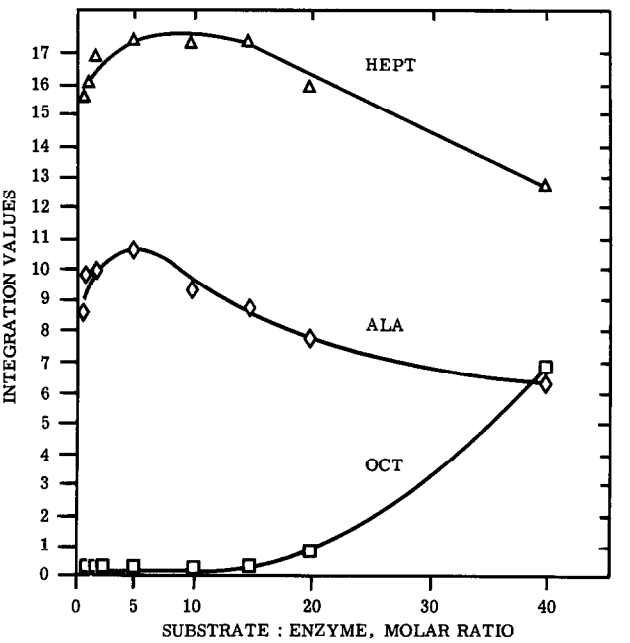

Fig. 1. The octapeptide, heptapeptide, and alanine pattern resulting from the proteolysis of 0.5 micromoles of the oxidized B chain of insulin per ml by varying amounts of TPCK-trypsin. Proteolysis was conducted for 1 min at 30° and pH 8. Integration values were calculated from amino acid analyzer charts. One ml of sample was analyzed after adding an equal volume of 0.2 N HCl to the reaction solution to prevent further cleavage.

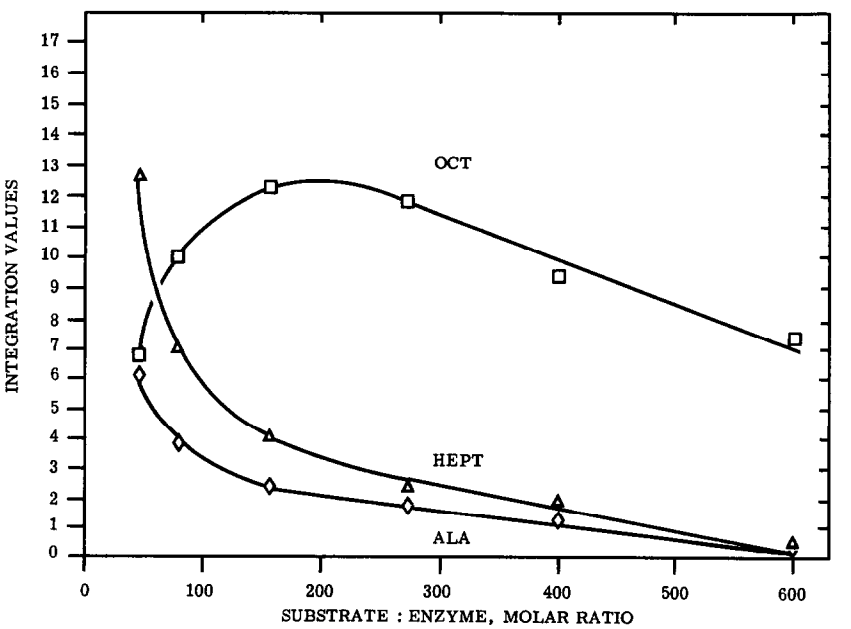

Fig. 2. Contracted substrate:enzyme ratio scale showing the effects of smaller amounts of TPCK-trypsin on the peptide and alanine pattern resulting from the protoelysis of 0.5 micromoles of the oxidized B chain of insulin per ml for 1 min at 30° and pH 8. Integration values were obtained as described in Fig. 1.

Autolysis of TPCK-trypsin during one minute incubation was determined by NPGB titration (9) in a manner comparable to that of B chain cleavage except that 100 µl of pH 8 buffer in place of buffered substrate was added to 100 µl of TPCK-trypsin. The reaction was stopped by NPGB in place of 0.2 N HCl. Three ml of pH 8.3 buffer (part of the titration procedure) were added to 50 µl of 0.01 M NPGB 20 sec before any enzymatic reaction was stopped by adding the buffered NPGB. Titrations of incubated buffered TPCK-trypsin were alternated with titrations of the enzyme in 1 mM HCl. In ten titrations, with the pH 8 buffered TPCK-trypsin in the same concentrations as in cleavage experiments with S:E = 1 (0.5 micromoles ml^{-1}) average autolysis in one minute was 2.8%, which is within the range of experimental error. In the same series of experiments a 10% decrease in the provided TPCK-trypsin in 1 mM HCl yielded titration values in good agreement with the amount of enzyme employed.

Attempts were made to measure the residual uncleaved B chain. TPCK-trypsin alone (conditions as in cleavage experiments except that 0.2 N HCl and then pH 8 buffer were added to TPCK-trypsin) emerged from the chromatographic column as a sharp peak. The B chain alone (conditions as in cleavage experiments except that 1 mM HCl substituted for TPCK-trypsin) emerged with a considerably broader base centered at essentially the same elution volume as the TPCK-trypsin. In each cleavage experiment, a single symetrical peak was observed. With pH and pyridine gradients one third as great as usual, the seeming homogeneity of the peaks did not change. When TPCK-trypsin and B chain were combined without pH 8 incubation (0.2 N HCl added to the reaction tube prior to the reactants producing a final pH of 1.2 with no octapeptide, heptapeptide, nor alanine formed) a single sharp peak also resulted with S:E ratios of 20 or less. The integration (chromogenic) values of the common peaks of unincubated (at pH 8) enzyme plus substrate were considerably less than the sums of the chromogenic values of the reactants alone. But the weight fractions of total TPCK-trypsin (active plus inactive enzyme) in the enzyme plus substrate combinations agreed well with the corresponding integration fractions (Table I). In the very low

TABLE I

WEIGHT AND CHROMATOGRAPHIC INTEGRATION VALUES OF THE OXIDIZED B CHAIN OF INSULIN MIXED WITH BUT UNCLEAVED BY TPCK-TRYPSIN

S:E molar ratio[a]	Total trypsin[b] mg/ml	Insulin B chain mg/ml	Trypsin / Trypsin + B Chain Weight	Trypsin / Trypsin + B Chain Integration[c]
1	18.74	1.83	.911	.912
2	9.37	1.83	.837	.821
5	3.75	1.83	.672	.650
10	1.87	1.83	.505	.528
15	1.25	1.83	.406	.426
20	.937	1.83	.339	.331

[a] Substrate:active enzyme molar ratio.
[b] Active plus inactive TPCK-trypsin (63.5% active).
[c] Enzyme plus substrate formed a single peak.
Experimental conditions were as described in Fig. 1 except that the enzyme and the substrate were added to 0.2 N HCl (in place of the reverse) to prevent cleavage.

S:E range, the weight of the enzyme was much greater than even the initial weight of the substrate (Table I). The accuracy of estimating changes in the residual substrate components of the enzyme plus substrate elution peaks of cleavage experiments was not considered to be adequate for meaningful quantitation.

In cleavage experiments with high enzyme concentrations, minor small chromatographic peaks appeared that matched peaks obtained with the corresponding enzyme preparation alone. No peaks were detected that could not be attributed to either the usual cleavage products or enzyme impurities.

DISCUSSION

A short reaction time (with the enzyme concentration as the only variable) was used in this initial study in order to further examine the product pattern over an extended range with particular attention to the details of the pattern with low S:E ratios. Longer reaction times would be expected to shift the curves of Figs. 1 and 2 to the right or the observed patterns might be expected with proportionately increased reaction times and decreased enzyme concentra-

tions. The product pattern varied from all octapeptide at a high S:E ratio to all heptapeptide and alanine at a range of low S:E ratios. As the enzyme concentration increased and the saturation rate of arginyl bond cleavage was reached, the octapeptide was converted to heptapeptide and alanine as rapidly as the octapeptide formed. The production of large amounts of heptapeptide and alanine in the absence of octapeptide occurred over at least a ten-fold range of enzyme concentrations. In principle, similar events would be expected in general in limited proteolysis in which bond cleavages occur at different rates resulting in products that serve as subsequent substrates. Whether given enzymes and substrates (including enzymes as substrates) reach local subcellular conditions in which some cleavage rates are maximal might be difficult to determine without destroying the original cellular conditions.

The elution of TPCK-trypsin and B chain as a single sharp peak suggests the formation of a somewhat stable complex under the reaction and/or chromatographic conditions and high enzyme concentrations.

Autolysis of the enzyme was considered as a possible cause of the reversal of the heptapeptide and alanine curves at high enzyme concentrations even though the octapeptide curve remained as zero (Fig. 1). However, autolysis in one minute was not found to be significant with the enzyme alone at pH 8 and 30° in the same concentration as in cleavage experiments with S:E = 1. The decrease in octapeptide and its final disappearance is considered to be, in large part, due to its conversion to heptapeptide and alanine; however, prior to reaching zero, the octapeptide decreased more rapidly than the heptapeptide increased. Peptide bond formation with high enzyme concentrations deserves investigation. It would be of interest if the heptapeptide or octapeptide joined the B chain desoctapeptide under the experimental conditions. Since the B chain components of the substrate plus enzyme chromatographic peaks were overshadowed by the enzyme components of the peaks, other experimental approaches to the quantitation of the substrate (or modified substrate) will be employed. It is not assumed that conditions such as pH 8 and 30° were optimal.

The differential or nondifferential effects of various inhibitors on cleavage patterns can be further examined with the aid of this background. Perhaps greater interest lies in kinetic and thermodynamic considerations of uninhibited subcellular limited proteolysis of various bioregulators and appropriate models.

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