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IDENTIFICATION OF HISTIDINE IN THE ACTIVE CENTER OF CHYMOTRYPSINS FROM A REPTILE AND A FISH

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SUMMARY

Chymotrypsin preparations from pancreatic tissue of two lower vertebrates, the turtle *Pseudemys elegans* and the teleost fish *Thunnus secundodorsalis*, were reacted with [*carbonyl*-^{14}C] tosyl-L-phenylalanine chloromethyl ketone. The alkylated protein was oxidized and hydrolyzed, and the labeled products were identified and measured in the effluent of an amino acid analysis chromatographic system. The results show that this inhibitor, which is known to selectively alkylate histidine-57 of bovine chymotrypsin A at N-3, alkylates in each of these species also only one histidine residue at N-3.

From this and other evidence, it is concluded that the active center of chymotrypsins is basically identical in structure throughout the vertebrates from the fish to the mammals.

INTRODUCTION

As shown in previous reports[1–3], enzymes generally similar in substrate specificity to bovine chymotrypsin have been found in the pancreas of all vertebrates so far tested, and these enzymes also exhibit two of the specific active center reactivities of the bovine enzyme. These are the reactions with near stoichiometric amounts of DFP and of *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK). The reaction with DFP is diagnostic, from many known examples[4], for an active center serine residue; the reaction with TPCK has been established[5] for bovine chymotrypsin A, where it alkylates histidine-57 (refs. 6 and 7). We report here evidence that TPCK, in the reaction with chymotrypsins from two lower vertebrates, similarly alkylates a histidine residue, and as in bovine chymotrypsin, attacks exclusively at the N-3 of the imidazole.

Abbreviations: TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLysCK, α-*N*-tosyl-L-lysine chloromethyl ketone.

MATERIALS AND METHODS

Materials

[1-^{14}C]L-Phenylalanine (radiochromatographically pure) was obtained from the New England Nuclear Corp., and L-phenylalanine from the Nutritional Biochemical Corp. Bio-gel P-10 (50–150 mesh) was from Bio-Rad Laboratories. Other materials were as specified previously[3]. The partly purified chymotrypsinogen from *P. elegans* used was that described[3], up to the stage where the first CM-cellulose chromatography would be applied. The chymotrypsin from the pyloric ceca of the tuna was purified to the stage described previously[3].

Synthesis of [carbonyl-^{14}C]TPCK

[1-^{14}C]L-Phenylalanine was mixed with L-phenylalanine to a specific activity of 0.130 mC/mmole, and was used in a modification of the TPCK synthesis of SCHOELLMAN AND SHAW[5]. It was converted[8] to *N*-tosyl-L-phenylalanine (recrystallized from 60% ethanol; m.p., 163° corr.). The acid chloride[9] was formed (white crystals; m.p., 129° corr.; in 76% yield). Reaction with diazomethane was as described[5], but only 1.66 equiv diazomethane (standardized against benzoic acid in ether) were used. The final product was recrystallized twice from ethanol, giving colorless needles (49% yield; m.p. 106–109° corr., alone and in mixture with authentic TPCK). Infrared spectra (in KBr wafers, read on a Perkin–Elmer 337 spectrophotometer) were identical over the 2.5–25 μ range for the [2-^{14}C]TPCK and authentic TPCK, as were their ultraviolet spectra: the latter were used in determining the specific activity of the product, 0.130 mC/mmole.

Amino acid analyses

Hydrolysis, analysis, and determination of ^{14}C-labeled products in a flow-cell scintillation system attached to the amino acid analyzer, were all procedures described in detail by GOREN, GLICK AND BARNARD[10].

Other methods

The performic acid oxidation medium was that of HIRS[11]. Other methods not specified were as described elsewhere[3,10].

RESULTS

Reaction of a turtle chymotrypsin with [^{14}C]TPCK

Chymotrypsinogen prepared[3] from *P. elegans* was fully activated, at a 4 mg/ml protein concentration, by bovine trypsin. An excess of the trypsin inhibitor α-*N*-tosyl-L-lysine chloromethyl ketone (TLysCK) was then added, such that after 23 h at 4° in the activation buffer (diluted 1:1 with water; pH 7.7), the total activities of the turtle trypsin present and of the added bovine trypsin were virtually extinguished. The chymotrypsin activity remained maximal. Most of the TLysCK was removed by dialysis against the same buffer (3 baths, over 6 h, at 4°) without loss of activity. The reaction with [2-^{14}C]TPCK was conducted in this solution, adjusted to pH 7.2 (Fig. 1).

After 92% inactivation, the protein solution was freeze-dried and filtered on a column of Bio-Gel P-10 (7.5 cm × 1 cm) in 0.01 M acetic acid, separating a single,

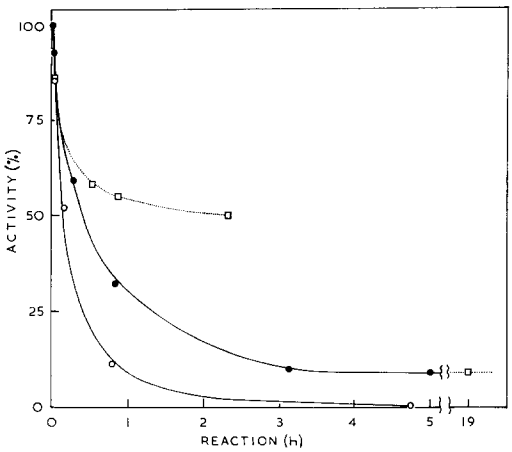

Fig. 1. Reaction of chymotrypsins with [2-^{14}C]TPCK at low inhibitor/enzyme ratios at pH 7.0 (25°). These were the specimens subsequently used for the analyses in Table I. The molar ratio of TPCK to chymotrypsin (measured as equivalents of the bovine enzyme, by *N*-benzoyl-L-tyrosine ethyl ester activity) was 4 for bovine α-chymotrypsin (○—○) at pH 7.0, 4 for tuna chymotrypsin (●—●) at pH 7.0, and 5.6 for turtle *(P. elegans)* chymotrypsin (□— — —□) at pH 7.2 initially and pH 7.0 finally. Each solution contained 3% methanol (v/v). The final activity shown for the turtle enzyme did not change after a further 12 h, when the protein was isolated.

labeled-protein peak centered at 7.0 ml, followed by the reagent peak. The protein was freeze-dried, dissolved in 0.5 ml formic acid, and oxidized with performic acid (Table I). It is known[6] in the case of a peptide from TPCK-inactivated bovine chymo-

TABLE I

OXIDATIONS AND ANALYSIS OF CHYMOTRYPSINS ALKYLATED BY [^{14}C]TPCK

The TPCK bound per mole enzyme was measured by counting the ^{14}C-labeled protein after gel filtration; the moles of enzyme were calculated by assuming an initial specific enzymic activity equal to that of bovine chymotrypsin A. ^{14}C recovery was determined, relative to the ^{14}C content of the oxidized protein, in the chromatogram (or its peak) run on the 60-cm column of the Beckman amino acid analyzer. Each analysis was performed twice, with concurrent results.

Species	*Moles TPCK bound per mole enzyme*	*Oxidation*			*^{14}C recovery (%)*	
		Protein (mg/ml)	*Temp.*	*Period (h)*	*In total hydrolysate*	*In N^3-carboxymethylhistidine peak*
Tuna	1.5	11	−5 to −7°	4.25	67	67
Turtle *(P. elegans)*	1.0	10	−8°	4.25	49	45
	1.2	1.2	+1°	8	45	41
Cow	1.2	10	−5 to −7°	4	36	35
		10	+1°	4	53	50
		2.5	+1°	8	56*	52

* When an aliquot of this hydrolysate was run on the 15-cm column (for basic amino acids) of the analyzer, 100% of the ^{14}C content was recovered in a single peak at the front position where the acidic and neutral amino acids emerged. The 44% of the ^{14}C not accounted for in the 60-cm column chromatogram is, therefore, a nonbasic product anomalously retained on that column. The chromatogram in the latter case was run for 1 h beyond the position where phenylalanine emerged, but no additional ^{14}C peak was eluted.

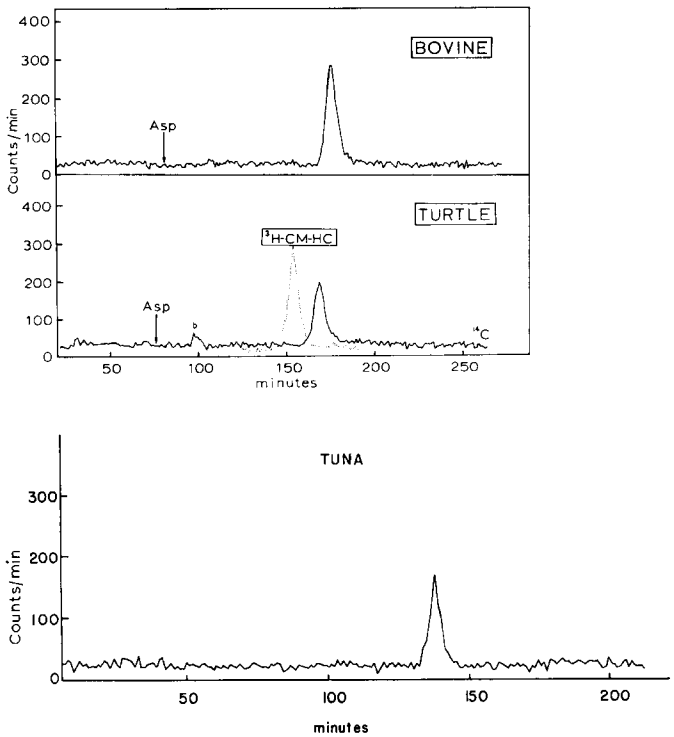

Fig. 2. Flow-cell chromatography of the hydrolysates on the 60-cm amino acid analyzer column[10]. ^{14}C (counts/min) was counted (———) continuously throughout the runs (past the point shown, to beyond the position of phenylalanine, with no further peaks being seen). The precise conditions applied varied between the runs within the limits described elsewhere[10], accounting for the different elution positions of the main peak, which in each case was shown to be N^3-carboxymethylhistidine by use in parallel chromatograms (not shown) of a ^3H-labeled marker[10] of this compound and of S-[^{3}H]carboxymethyl-homocysteine (^{3}H-CM-HC) known just to precede the former compound, as shown (· · · · · ·) in the case of the turtle. The turtle was *P. elegans*. Asp represents the position of [^{14}C]aspartic acid added in parallel runs as an internal calibration standard. The minor peak b occurred only in the turtle case, and is unidentified.

trypsin, oxidized on filter paper with performate, that a rearrangement and cleavage occur yielding carboxymethylhistidine. The performic acid was removed by addition of water and freeze-drying, repeated twice. The oxidized protein was hydrolyzed in 6 M HCl, (110° ± 1°, for 20 h), and the hydrolysate was examined on the amino acid analyzer with flow-cell counting of radioactive peaks[10]. Almost all of the radioactivity detected was in a single peak, corresponding to N^3-carboxymethylhistidine (Fig. 2; Table I).

Reactions of tuna and bovine chymotrypsins with TPCK

The partly purified chymotrypsin from tuna[1] was reacted with [2-^{14}C]TPCK (Fig. 1) in 0.01 M Tris–acetate (pH 7.0). After 96% inactivation, the alkylated protein was separated from all the reagent by gel-filtration as for the turtle enzyme, and then oxidized (Table I). Subsequent treatment was as for the turtle enzyme. In the analysis, virtually all the radioactivity was found in the N^3-carboxymethylhistidine peak (Fig. 2; Table I).

Bovine α-chymotrypsin was reacted similarly with [2-^{14}C]TPCK (Fig. 1). At 99% inactivation, the same procedure was applied. Again, all the radioactivity detected in the analysis was in the N^3-carboxymethylhistidine peak (Fig. 2). This product was observed previously[6] by other methods in the case of bovine α-chymotrypsin.

The yield of carboxymethylhistidine, relative to the ^{14}C present in each alkylated protein preparation, was less than quantitative, and it did not increase when the oxidation was prolonged (Table I). Since all the cystine present was found to have become converted to cysteic acid after the performate treatments above −8°, these oxidations appear to be efficient. The unrecovered ^{14}C appeared to be in a product retained on the 60-cm column of the amino acid analyzer (see Table I). It was concluded that this probably reflects a greater tendency to side reaction in the oxidation of the alkylated protein as compared to small peptides.

The incorporation of reagent residues into the inactivated protein was measured (Table I) after the Bio-Gel filtration step in each of the three cases, but these values are only approximate due to impurity and lack of knowledge of the exact specific enzymic activity (on *N*-benzoyl-L-tyrosine ethyl ester as substrate) of these chymotrypsin preparations.

DISCUSSION

The reaction with TPCK, when determined with the isotopic reagent, is seen to occur very efficiently (Table I), and to mofigy only one residue in the reptile enzyme and in the fish enzyme. This confirms the conclusions drawn from inactivation rates.

If attack by TPCK occurred at any residue in addition to the one histidine, the products were present in such small amounts that they could be ignored. Further, no significant attack occurred at the N-1 of that histidine, since N^1-carboxymethylhistidine residues are readily and quantitatively measured when hydrolysates of the protein are analyzed by the flow-cell method used[10].

Hence, the reaction with TPCK of chymotrypsin from the pancreas of one reptile and one teleost fish species is now seen to take place at an equivalent site to that in the bovine enzyme, *i.e.*, at N-3 of one histidine residue. Since the nucleophilicity at N-1 in free imidazoles and histidine is not greatly different[13] to that at N-3, the maintenance of this complete selectivity and the enormous efficiency of the reaction (Fig. 1) supports the conclusion[3] that the essential structure of the active center in chymotrypsin is the same throughout the vertebrate series. This generalization from the cases tested here is based upon the facts that this histidine reactivity is now established for each case examined, drawn from three different vertebrate classes, also that a comparable inactivation with TPCK or *N*-tosyl-L-leucine chloromethyl ketone (though at a site not identified) has also been demonstrated for representatives of many vertebrate groups[2,3], and that the DFP reactivity characteristic of the active center serine is simultaneously present there[2].

The reaction described here is being used to aid the isolation and characterization of labeled peptides from these chymotrypsins. It will be of considerable interest to compare the sequences around the active center histidine in some lower vertebrate cases with the corresponding sequences known for other chymotrypsins, those from the cow[7,14] and the pig[15] being the only ones known so far.

NOTE ADDED IN PROOF (Received March 26th, 1969)

In subsequent work by W. Möckel and E. A. Barnard, to be reported in detail in a further paper, an isolation has been made of a peptide carrying the single alkylated histidine residue in a TPCK-inactivated derivative of the chymotrypsin from the turtle *Chelydra serpentina*. It has been found that this histidine, too, is substituted at the N-3 position. The most probable sequence of this peptide has been found to be: Ala-Ala-His-Cys-Gly-Val-Thr-Thr-Ser, identical to that at positions 55–63 in bovine chymotrypsins A and B.

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