ISOLATION AND CHARACTERIZATION OF THE ALKYLATED HISTIDINE FROM TLCK INHIBITED TRYPSIN

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Recently Shaw, Mares-Guia, and Cohen (1965) have shown that L-1-chloro-3-tosylamido-7-amino-2-heptanone (the chloromethyl ketone derivative of N^{α}-tosyl-L-lysine, TLCK) inhibited trypsin stoichiometrically in a 1:1 molar ratio Amino acid analysis of the inhibited trypsin showed a loss of one histidine residue when compared to the control. Positive evidence for histidine as the site of alkylation of trypsin by TLCK is now presented with a description of the isolation of the histidine derivative (I) as a crystalline dipicrolonate. The reaction occurs predominantly at the 3 position of the imidazole ring since only 3-carboxymethylhistidine has been found in the hydrolysate of trypsin inhibited by TLCK and subjected to performic acid oxidation.

Preliminary work indicated that p-toluenesulfonamide was being released from TLCK-inhibited trypsin and that this instability was greatly diminished by reduction of the keto group in the inhibitor residue with sodium borohydride. The isolation described below takes advantage of this property and of the high affinity of the histidine derivative (I) for a sulfonated polystyrene resin

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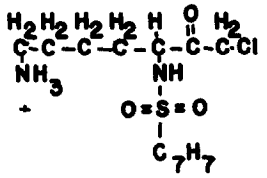
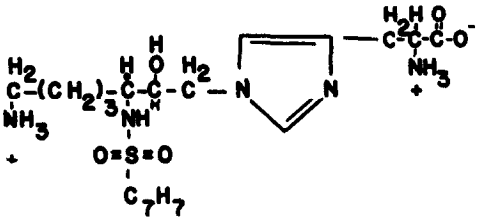
which permitted its isolation from the other amino acids in the hydrolysate of inhibited enzyme. It was then further purified on a subsequent column of a carboxylate resin.



TLCK



I

Isolation of the Alkylated histidine derivative (I)

Trypsin inhibited with tritiated TLCK as described (Shaw et al., 1965) was reduced with sodium borohydride in 8M urea at 37° C using 0.5% solutions of protein and reagent (Moore et al., 1958). The reduced inhibited enzyme (3.5 g.) was refluxed with about 35 times its weight of 6N hydrochloric acid for eighteen hours and, following removal of the acid under reduced pressure, the hydrolysate was applied to a column (0.9 x 15 cm.) of Aminex-MS (Bio-Rad blend Q-15) in the pyridinium form. Elution of the amino acids was carried out at 50° C with a pyridinium acetate buffer of pH 5.28 (25 ml of glacial acetic acid, 56.4 ml of pyridine, 2000 ml of deionized water). All of the amino acids eluted with 300 ml of buffer along with 35% of the radioactivity applied which is considered to have arisen by degradation of the inhibitor since the alkylated histidine itself cannot be removed from this column under such conditions. (In fact, it is not removable by a solution containing 30% pyridine-10% glacial acetic, by pyridine, or by 6N hydrochloric acid). The histidine derivative was stripped from the column at room temperature by elution with 4N ammonium hydroxide containing 10% methanol; 55% of the radioactivity was recovered. The ammoniacal pool was taken to dryness under reduced pressure. The residues from two such runs were combined in pyridine acetate buffer (5 ml. starting buffer, Fig. I) and applied to column (0.9 x 60 cm.) of Amberlite CG-50 in the pyridinium

form. A pyridinium acetate gradient then provided a major peak (Fig. I) containing 85% of the radioactivity applied to this column. Paper chromatography on Whatman 3MM, ascending, showed the presence of a single radioactive material with Rf = 0.54 in butanol, pyridine, water (60:60:60, V/V/V), Rf= 0.15 in butanol, acetic, water (120, 30, 50, V/V/V), and Rf = 0.64 in phenol, water (160:40, W/W). There was a trace of another ninhydrin positive material that persisted through picrolonate formation.

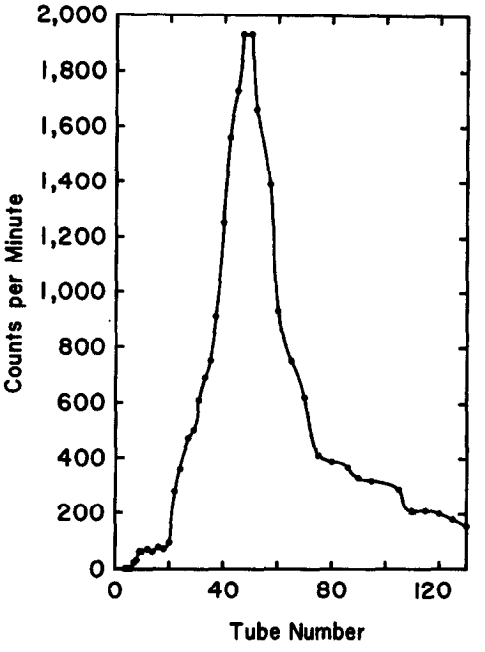


Fig. 1 Chromatographic elution of the alkylated histidine residue on a column (0.9 x 60 cm.) of Amberlite CG-50. A linear gradient was used: 800 ml. of 1% pyridine - 0.1% of glacial acetic acid in the mixing chamber, and 800 ml. of 10% pyridine - 5% glacial acetic acid in the reservoir. The fraction volume was 12.3 ml. The flow rate was 57 ml. per hour. 0.2 ml. aliquots were plated for radioactivity measurements.

The eluate in the peak was pooled and, after removal of the solvent, the residue was taken up in water (2 ml.) and adjusted to pH 7; saturated

aqueous picrolonic acid was added gradually until precipitation ceased. After two hours at room temperature, the precipitate was collected by centrifugation, washed with ice water, and dried in a vacuum desiccator over anhydrous calcium chloride; 37% of the theoretical yield, 110 mg., m.p. 154-158° C, was recovered as a first crop of crystals. This material appeared to be trihydrate of the dipicrolonate since at 100° C and 2 mm pressure it lost 5.5% by weight (theory for trihydrate = 5.2%). For material dried in this manner the elemental analysis was:

$C_{20}H_{31}N_5O_5S \cdot 2C_{10}H_8N_4O_5$ calcd: C, 48.9; H, 4.79; N, 18.5; S, 3.26
found: C, 48.3; H, 4.38; N, 17.8; S, 3.45.

Formation of 3-carboxymethylhistidine in TLCK-inhibited trypsin on performic acid oxidation

Stevenson and Smillie (1965) have observed that certain ketomethyl histidine derivatives (such as peptides from TPCK-inhibited chymotrypsin) are oxidized when present in paper chromatograms exposed to performic acid vapors; 3-carboxymethylhistidine was detected in hydrolysates of these peptides. TLCK-inhibited trypsin was treated with performic acid in solution by the method of Hirs (1956) with prolongation of the oxidation to eight hours to ensure complete oxidation of cystine to cysteic acid. This was done to achieve unambiguous identification of 3-carboxymethylhistidine which under the standard conditions of automatic amino acid analysis of Spackman, Moore, and Stein (1958) emerges at the same place as cystine. (The more vigourous oxidative conditions of Moore (1963) destroy histidine and its derivatives and cannot be used.) 3-Carboxymethylhistidine was obtained from TLCK-inhibited trypsin as summarized in Table I.

3-Carboxymethylhistidine can also be unambiguously indentified by operation of the analyser with a slightly more acidic buffer than usual, pH 3.21 ± .02 (vs 3.25), resulting in relatively more retardation of cystine than 3-carboxymethylhistidine and an adequate degree of resolution. The position

of 3-carboxymethylhistidine was checked with authentic material (Crestfield et al., 1963). Under these conditions, 1-carboxymethylhistidine was almost completely separated from proline.

TABLE I

3-carboxymethylhistidine from performic acid oxidized TLCK-inhibited trypsin

AMINO ACID	RESIDUES FOUND			
	TRYPSIN	TRYPSIN INHIBITED BY TLCK		
		Untreated	performic acid oxidized	
			Borohydride reduced	Untreated
Histidine	2.9	2.0	2.1	2.2
Cystine	12.4	11.6	0	0
Cysteic Acid	0	0	11.6*	12.1*
3-CM Histidine	0	0	0	0.55**
1-CM Histidine	0	0	0	0

* Corrected for 90% recovery of cysteic acid (Hirs, 1956).

** The carboxymethylhistidine peak was calculated by the Net Total Absorbance method using our glycine color constant which is 21.1.

DISCUSSION

During this work and concurrent studies on TPCK-inhibited chymotrypsin (Veith, 1965) an unexpected lability of the p-toluenesulfonamido group in the inhibitor was encountered. With tritiated TPCK and TLCK prepared by the Wilzbach technique, a large proportion of the label is taken up by this aromatic residue. Continual losses of radioactivity from inhibited enzymes was observed and found to be due to release of p-toluenesulfonamide by an unknown mechanism. After borohydride reduction of the keto group, this side reaction was largely prevented

and an isolation procedure was successfully devised for the alkylated histidine derivative formed by the action of TLCK on trypsin. The high affinity of this residue for certain resins as well as its instability are properties that will have to be taken into account in structural studies of proteins modified by TLCK.

The borohydride reduction of the inhibitor residue from a keto to an alcohol, however, interferes with the application of performic acid oxidation for the cleavage of the residue to a carboxymethyl group (Table I). Only in the keto form was the oxidation successful leading to the characterization of 3-carboxymethylhistidine and permitting the assignment of the histidine substitution as in (I). The yield of 0.55 residues of 3-carboxymethylhistidine* is considered satisfactory in view of the fact that column purified trypsin bound only 0.8 residues of TLCK (Shaw, Mares-Guia, Cohen, 1965) and probably still contained inactive enzyme. In addition, the oxidative cleavage may occur on the other side of the keto group and only one of these modes leads to 3-carboxymethylhistidine. Finally, the optimal conditions of oxidation remain to be worked out. It is of interest that no 1-carboxymethylhistidine was detectable under conditions very favorable for its resolution as described above.

Substitution at the 3-position of histidine on inactivation of chymotrypsin (Stevenson and Smillie, 1965; Veith, 1965) and trypsin by chloromethylketone substrate derivatives is in contrast to the findings on iodoacetate inactivation of ribonuclease in which N-1 (of histidine 119) was the chief site of alkylation (Crestfield, et al., 1963).

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* Inagami (1965) has studied the alkylation of trypsin by iodoacetamide in the presence of alkylguanidines and found 3-carboxymethylhistidine on hydrolysis. However, since seven moles of iodoacetamide reacted per mole of trypsin, the significance of this observation is difficult to assess.

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