MOLECULAR WEIGHT DETERMINATION OF THE CHYMOTRYPSIN-LIKE PROTEASE OF THE LARVA OF THE HORNET, *VESPA ORIENTALIS*, BY AFFINITY LABELING OF THE ACTIVE CENTER

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1. Introduction

Previously we determined the molecular weight of the hornet chymotrypsin by physico-chemical methods [1]. The molecular weight was found to be about 13 800. This molecular weight is much lower than the other ones known of the chymotrypsins isolated from vertebrates or other invertebrates. However, the low molecular weight might be due to an unusual structure of the enzyme. Using affinity labeling technique, one can determine the minimal molecular weight of an enzyme. Therefore we have used active site directed reagents known for endopeptidases. Bovine chymotrypsin reacts with DFP, PMSF and the chloroketones derived from phenylalanine stoichiometrically in a ratio of 1:1, i.e. one mole reagent is bound to one mole of the active center of the enzyme [2,3].

2. Materials and methods

Hornet chymotrypsin was purified to an homogenous stage as described [1]. The enzyme activity was measured photometrically at 405 nm using glutaryl-L-phenylalanine-*p*-nitroanilide as substrate [4]. For inhibition the enzyme (0.32 mg/ml) was incubated with an excess of the labeled inhibitor in Na–phosphate buffer pH 7.4 at 25°C for 6 hr. After this incubation no enzyme activity was detectable. The samples were dialysed exhaustively against 0.1 Na–phosphate buffer pH 7.1 at 4°C to remove all unbound inhibitor. 50 µl of the dialysed samples were counted in 5 ml of Pinaud's [5] scintillation solution using the liquid scintillation counter Tracer-Lab. The protein concentration was determined by the Lowry method [6]. [^{14}C]ZPCK was synthesized according to Shaw [7] with small modifications ([^{14}C]ZPCK m.p. 102–104°C, lit. 99–102°C, specific radioactivity 0.25 mC mmole). For amino acid analysis, approx. 0.3 mg of the protein was hydrolyzed in 6 M HCl for 20 hr in evacuated sealed tubes at 108°C. The analyses were performed on the Bio-Cal aminoacid analyzer model BC 200 according to the method of Spackman [8]. [^{35}S]PMSF and uniformly labeled 1-[^{14}C]phenylalanine were purchased from Radiochemical Centre, Amersham, PMSF and TPCK were obtained from Serva, Heidelberg. All other chemicals were analytical grade reagents from E. Merck, Darmstadt.

Abbreviations: DFP diisopropyl fluorophosphate, PMSF phenylmethanesulfonylfluoride, ZPCK benzyloxycarbonyl phenylalanine chloromethyl ketone, TPCK tosylamido-2-phenylethyl chloroketone.

3. Results and discussion

3.1. *Reaction with PMSF*

It is well known that PMSF sulfonates the essential residue in the active center of bovine chymotrypsin which results in a complete loss of enzymic activity [2]. Also incubation of hornet chymotrypsin with this inhibitor results in a complete inactivation of this

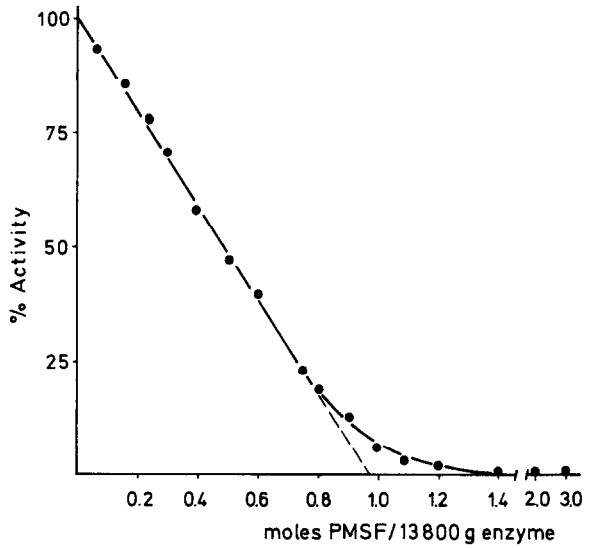

Fig. 1. Titration of hornet chymotrypsin with PMSF. Enzyme incubated at pH 7.4, 25°C for 1 hr prior to assay.

enzyme. This observation may indicate that hornet chymotrypsin is inactivated in a similar manner to bovine chymotrypsin. A titration of hornet chymotrypsin (0.56 mg/ml) with increasing amounts of PMSF is shown in fig. 1. The enzyme is incubated at pH 7.4 and 25°C for 1 hr prior to measuring residual activity. The loss of enzyme activity is proportional to the amount of inhibitor up to 20% residual activity. By extrapolation the enzyme must be completely inhibited when one mole PMSF is added to 13 500 g enzyme. Since this value is close to the molecular weight as determined previously [1], the reaction appears to have a 1:1 stoichiometry.

However, the determination of the amounts of inhibitor incorporated in the enzyme is a better criterion of stoichiometry. Therefore the enzyme was treated with [^{35}S]PMSF as described under Materials and methods. Table 1 shows the degrees of labeling. When labeling the enzyme with [^{35}S]PMSF, a mol. wt. of about 13 100 was obtained related to one essential serine residue.

3.2. *Reaction with ZPCK*

The chloroketones derived from phenylalanine alkylate the essential histidine residue at the active center of bovine chymotrypsin [2,3]. Since hornet chymotrypsin was also rapidly inhibited by ZPCK or

Table 1
Labeling of hornet chymotrypsin by [^{35}S]PMSF and [^{14}C]ZPCK

	Inhibitor conc. nmole/ml	Inhibitor conc.* after dialysis nmole/ml	Mol. wt., when one mole incorporated	Mole inhibitor mole enzyme (13 800)
Enzyme inhibited by [^{35}S]PMSF for 6 hr at pH 7.4 protein concentration 0.32 mg/ml	198.0	21.88	14600	0.97
	99.0	24.46	13100	1.08
	65.5	27.10	11800	1.19
	49.5	24.45	13100	1.08
			13150	1.08
Enzyme inhibited by [^{14}C]ZPCK for 6 hr at pH 7.6 protein concentration 0.32 mg/ml	252.0	24.80	12900	1.09
	126.0	24.00	13300	1.06
	63.0	18.10	17600	0.80
	31.0	21.92	14600	0.97
			14600	0.98
PMS–hornet chymotrypsin incubated with ^{35}S-PMSF	256	0.9		0.04
PMS–hornet chymotrypsin incubated with ^{14}C-ZPCK	314	0.0		0.0

* Average of 4 determinations

Table 2
Amino acid composition of native hornet chymotrypsin and modified hornet chymotrypsin by ZPCK or TPCK

Amino acid	Native enzyme	Enzyme modified by	
		ZPCK	TPCK
Aspartate	13.0	12.9	12.8
Threonine	6.6	6.7	6.7
Serine	9.2	9.6	9.2
Glutamate	10.2	10.3	10.1
Proline	6.0	6.2	6.1
Glycine	13.0	13.1	13.0
Alanine	5.9	6.0	6.0
Valine	10.0	10.1	10.3
Methionine	0	0	0
Isoleucine	8.1	8.3	8.6
Leucine	10.1	10.1	10.1
Tyrosine	3.8	3.9	4.1
Phenylalanine	2.9	2.9	2.9
Histidine	3.6	2.6	2.7
Lysine	8.2	8.0	8.0
Arginine	4.5	4.3	4.4

Residues in 13 800 g enzyme, tryptophan and half-cystine not determined

TPCK, analogous experiments were carried out with [^{14}C]ZPCK as in the incorporation studies of PMSF. After dialysis, the protein contains one mole alkylating reagents per 14 500 g enzyme related one essential histidine residue. The incorporation experiments were confirmed by amino acid analyses of hornet chymotrypsin before and after inactivation by ZPCK or TPCK. The amino acid analyses are given in table 2. No differences in the amounts of the individual residues except for histidine are observed. The significant change of histidine is due to the modification by ZPCK and TPCK. Taking a mol. wt. of 13 800, one histidine residue has disappeared after reaction with these inhibitors. There is a decrease from four histidines residues per mole of native hornet chymotrypsin to three ones in the inactivated enzyme.

The results of the molecular weight determination by affinity labeling agree quite well with the mol. wt. of 13 800 obtained by ultracentrifugation, gelfiltration, and electrophoresis at different gel concentrations. Assuming a mol. wt. of 13 800, hornet chymotrypsin binds one mole [^{35}S]PMSF or [^{14}C]ZPCK to one mole enzyme. This result suggests there is one active center present in hornet chymotrypsin. However, assuming a mol. wt. of 24 500, a value found for mammalian chymotrypsins with one active center, hornet chymotrypsin binds 1.8 mole [^{35}S]PMSF or 1.7 mole [^{14}C]ZPCK, i.e., the enzyme would have two active centers. However, no evidence has been found in the kinetical studies yet that hornet chymotrypsin has two active centers. But the possibility of two active centers can not be completely excluded by these experiments.

Incubation of a completely inhibited hornet chymotrypsin by PMSF or TPCK with a 10-fold molar excess of [^{35}S]PMSF or [^{14}C]ZPCK for 10 hr shows no measurable rate of radioactivity after removing the inhibitor by dialysis. These results show that no inhibitor is unspecifically bound to the protein. To check our method the experiments were repeated in the same way with bovine chymotrypsin and we observed an incorporation of nearly one mole of [^{35}S]PMSF or [^{14}C]ZPCK to 24 500 g enzyme as also described in literature [2,3].

Together with our results previously obtained by physicochemical methods [1], hornet chymotrypsin must have a minimal mol. wt. of about 13 800, and this polypeptide chain posesses one active center.

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