MECHANISM OF CHYMOTRYPSIN HYDROLYSIS OF AN ESTER AND AN ANILIDE

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Received 14 May 1971

1. Introduction

The serine proteases (e.g. chymotrypsin, trypsin, subtilisin) cleave amino acid esters via an acylated enzyme intermediate [1]. The mechanistic pathway with amino acid amides, peptides, etc., has not been so clearly established, however, in that no acyl-enzyme intermediate has been found using these substrates. Studies based on partial blocking of the active site serine [2] or partitioning of the presumed acyl intermediate between competing nucleophiles [3] have suggested that amide, anilide, and peptide substrates do not have to be hydrolyzed via the acyl enzyme.

I have examined the loss of activity of chymotrypsin towards an ester substrate and an anilide substrate as a result of partial carbamylation of the active site serine [4]. This derivative of the enzyme is quite stable, and not subject to enzymatic deacylation, removing the ambiguity possible when blocking agents such as acetyl are used. In addition, the carbamyl moiety is small enough that it should produce minimum steric hindrance of the active site, as opposed to, e.g. the di-isopropyl-phosphoryl derivative [2].

2. Experimental

Chymotrypsin (3X crystallized, Worthington Biochemical Corp.) 40 mg/ml was reacted with 1.25 M potassium cyanate at pH 6.5, 30°, in a Radiometer pH-stat. The pH was held constant by addition of 1 N HCl. After 0, 10, 30 and 60 min reaction time, 0.5 ml portions were removed and quickly passed over a 20 X 1 cm column of G-50 Sephadex (Pharmacia) in 0.001 N HCl. The main peak of protein was collected in a total volume of ∿ 6 ml. Protein concentration was determined by spectrophotometry ($A_{282}^{1\%} = 20.75$ [5]). Enzyme normality was determined by reaction with *N-trans*-cinnamoylimidazole (CI) [6]. Esterase activity was measured by reaction with *N*-acetyl-L-tyrosine ethyl ester (ATEE), S_0 = 1 mM in 0.05 M phosphate buffer pH 7.2, following the decrease in absorbance at 237 nm [7]. The pseudo-first order rate constant C was measured and divided by the mg protein used in each assay for comparison purposes. Anilide activity was measured by the hydrolysis of glutaryl-L-phenylalanyl-*p*-nitroanilide (GPANA) [8] in 0.05 M phosphate buffer, pH 7.2. The rate of increase of absorbance at 410 nm was followed with S_0 = 1 mM. Results are given as ΔA_{410}/min/mg protein.

3. Results

In table 1 the results of these experiments are given. The most important point is that the enzyme loses activity towards both an ester and an anilide substrate in an exactly parallel manner. There is no suggestion that the carbamylated enzyme may still be able to catalyze hydrolysis of the anilide by some mechanism not involving an acyl-enzyme intermediate. This would seem to directly contradict the conclusions of Bresler et al. [2].

A second point of interest, though less germane to the immediate question, is that chymotrypsin does not lose as much activity as measured by CI titration (all-or-none assay) as it does by ATEE or GPANA assays. This is apparently due to heterogeneity of the enzyme species in the sample. Chymotrypsin was incubated with 1 M potassium cyanate, pH 6,5, 30°. Aliquots were withdrawn at various times and titrated

Table 1

Time of reaction (min)	CI titration		ATEE hydrolysis		GPANA hydrolysis	
	% Active [a]	Rel. %	C/mg protein	Rel. %	ΔA_{410}/min/mg	Rel. %
0	99.3	100	1.052	100	0.128	100
10	70.8	71	0.737	70	0.085	67
30	57.0	57	0.504	48	0.060	47
60	48.2	49	0.380	36	0.043	33

[a] % of the protein which is active enzyme, taking the molecular weight of chymotrypsin to be 25,000.

directly with CI. A plot of log (% CI activity remaining) versus time of reaction was obviously biphasic, with a component representing 29% of the initial enzyme losing activity with a rate constant $k = 0.086\ min^{-1}$, and the rest of the enzyme losing activity with a rate constant $k = 0.0045\ min^{-1}$. Similar plots were reported by Erlanger et al. [8] for the thermal denaturation of chymotrypsin.

4. Discussion

Bresler, Krutyakov and Vlasov [2] studied the relative activities towards ester and anilide substrates of trypsin in which the active site serine was acylated with various small aliphatic acids. Using the acetyl derivative, for instance, they found 6% remained of the activity of unmodified trypsin with N^{α}-carbobenzoxy-L-arginine ethyl ester as the substrate, while with N^{α}-carbobenzoxy-L-arginine-*m*-nitroanilide as the substrate they found that 37% of the initial activity remained. They interpreted this (and a number of similar results) to mean that the acyl-enzyme is not an obligatory intermediate in the hydrolysis of anilides and peptides by trypsin. Similar conclusions have been reached for chymotrypsin, based upon partitioning experiments [3].

The reasons for the discrepancy between my results and the conclusion reached by Bresler et al. [2] are not clear. The use of the acetyl moiety as a serine blocking agent makes possible some experimental artifacts due to deacylation which could render interpretation of their data difficult. It is known that small molecules can accelerate the deacylation of acetyl-chymotrypsin [9, 10]. Bresler et al. have apparently considered this possibility and stated that they carried out control experiments which ruled it out [2]. It is not possible to judge from their paper how firm this exclusion was.

The other significant difference between the two experiments is that with the acetyl blocking groups, a hydrophobic $-CH_3$ substituent is attached to the carbonyl carbon, while with the carbamyl blocking group this substituent is a polar $-NH_2$ group. If all other possible explanations of the results of Bresler et al. are ruled out, and the non-acyl enzyme pathway for anilide hydrolysis is confirmed, the blocking of that pathway by the presence of a polar carbamyl $-NH_2$ group would appear to be a significant and useful probe into the nature of this alternate mechanism. Another possibility, of course, is that significant mechanism differences exist between chymotrypsin (used here) and trypsin (used by Bresler et al.), at least with respect to anilide hydrolysis. I think it more likely, however, that the results reported here will help confirm that all substrates of serine proteases are hydrolyzed via an acyl enzyme intermediate.

Acknowledgement

I wish to thank Dr. E. Zeffren for stimulating discussions about this aspect of serine protease mechanism.

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