SUCCINYLATION OF THE AMINO GROUP OF ISOLEUCINE 16 IN δ-CHYMOTRYPSIN WITH RETENTION OF ACTIVITY

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Received 4 March 1971

1. Introduction

The mechanism of chymotryptic activity is thought to involve the amino acid residues Ser-195, His-57, and Asp-102 participating in a coupled 'charge-relay' system [1]. However, evidence has been presented for the possible participation of another group. This evidence includes proton uptake as a function of the binding of various substrates [2–4], the kinetics of the acylation reaction involving non-specific substrates [5, 6], the dependence of k_{cat}/k'_m on pH for various substrates [5] and of k_s on pH for amide hydrolysis [6, 7]. This group has a pK' of 8.5–9.0 and is thought to be the amino group of Ile-16 [8–11]. Conversion of the inactive zymogen to the active enzyme is said to be accompanied by the formation of an internal ion pair involving the carboxylate anion of Asp-194 with Ile-16 [12, 13]. Crystallographic [12–14] and circular dichroism studies [15] support the proposition that the functional behavior of the residue is to regulate the concentration of active site necessary for fruitful enzyme-substrate binding.

A significant role in the development of the ion-pair hypothesis has been played by amino group modification. Treatment of the amino group of Ile-16 with either nitrous acid [11] or acetic anhydride [9] results in a considerable decrease in enzymatic activity. However, potentiometric titration studies of the reaction between chymotrypsin and formaldehyde [16, 17] or diisopropylphosphofluoridate [18] have indicated that the Ile-16 amino group is not necessary for chymotryptic activity. Kinetic studies done with δ-chymotrypsin support this conclusion [19]. Conversion of the amino group, pK' 8.5–9.0, to an amidine group, pK' 11–12, with retention of specific activity has also been reported [20], although this modification would not completely obviate the formation of an ion-pair.

We wish to report that we have succeeded in blocking the amino group of Ile-16 with succinic anhydride creating a new negatively charged residue without major change in the specific activity.

2. Experimental

Acetylated chymotrypsinogen was prepared by the addition of 140 μl (2550 μmoles) acetic anhydride over a 60 min period to 1.0 g (40 μmoles) chymotrypsinogen in a 15 ml volume (0.15 M KCl, 0.02 M $CaCl_2$) at 2°. The pH was maintained at 7.9 with a Radiometer pH-stat. After addition of all the acetic anhydride, the pH was allowed to fall to 6.5 where the excess anhydride was hydrolyzed. Before reaction with acetic anhydride, the chymotrypsinogen was reacted at pH 7.5, 20° for 1 hr with diphenylcarbamyl chloride (1.1 molar excess over the residual chymotrypsin activity).

Rapid activation of the acetylated chymotrypsinogen to the active δ-enzyme was accomplished by the addition of one part trypsin to 50 parts zymogen at pH 7.5 and 2° for 210 min. The material was

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passed through Sephadex G-50 at pH 6.5 in the cold and the portion containing active enzyme was lyophilized. Resuccinylation of acetylated-δ-chymotrypsin (200 mg per 4 ml of 0.15 M KCl) was done at pH 7.75 and 2° with a 20-fold excess of solid succinic anhydride which had been recrystallized from acetic anhydride. After termination of the reaction (30–40 min), the pH was adjusted to 6.2 and the material dialyzed overnight against cold deionized water (3 × 10 l). Yields were about 70% of the starting material.

Potentiometric titrations were done as previously described [21]. Specific activities were determined using acetyl-L-tyrosine ethyl ester as substrate in a final concentration of 0.015 M at pH 8.0 and 20° in a solution of 0.15 M KCl. *Trans*-cinnamoylimidazole was used as the reagent for active site assays [22]. End group analysis was done by established procedures using phenyl isothiocyanate [23]. Protein concentrations were determined by using an $\epsilon^{1\%}_{282\ nm}$ of 20 and a molecular weight of 26,000.

Table 1
Effect of succinylation on acetylated-δ-chymotrypsin.

Experiment	Treatment with succinic anhydride	Specific activity[a]	%Active sites	Isoleucine Equivalents
1	–	138	80	0.98
	+	132	63	0.47
2	–	128	77	–
	+	113	60	0.38
3	–	124[b]	75	1.01
	+	108[b]	64	0.10

[a] The specific activity is reported as the specific catalytic rate constant, $k_{cat\ (sec^{-1})}$. It is uncorrected for active site concentration. Under the experimental conditions employed, K'_m for the acetylated-δ-chymotrypsin was 3.0×10^{-4} M and 5.2×10^{-4} M for the succinylated derivative.
[b] The assay system contained 0.05 M $CaCl_2$.

3. Results and discussion

It may be argued that modifications which replace the positively charged amino group of Ile-16 with another positively charged derivative need not interfere with the ion-pair bond to the negatively charged Asp-194 residue and thus have no effect on the proposed function of the Ile. This, however, would necessarily mean that the introduction of a large group had no steric effect on the enzyme active site. Further, a protein with a newly introduced group with pK' ∿ 12 should not show the typical bell-shaped kinetics with a non-ionizing substrate. This is, however, contrary to recent findings [20]. The introduction of a negative charge onto the Ile-16 amino group will eliminate any possible ion-pair bonding to a negatively charged carboxyl residue. If a positively charged amino group is necessary, then a direct loss of enzymatic activity should be seen proportional to the amount of negative charge introduced and the equivalents of Ile-16 lost.

Clearly, the data presented in table 1 does not support the necessity for a positively charged amino group. When acetylated-δ-chymotrypsin is treated with succinic anhydride a variable amount of new negative charge is introduced onto the Ile-16 as indicated by the equivalents of Ile recovered. However, the specific activities of the treated proteins are all high, much higher than would be expected if disruption of the ion-pair were to destroy enzymatic activity. Treatment of the starting protein with succinic anhydride reduces both the specific activity and the active site concentration by about 10–20% even when up to 90% of the Ile amino group had been blocked. Potentiometric titrations of the proteins from Experiment 3 indicated that the initial acetic anhydride treatment blocked all amino groups on the zymogen and treatment with trypsin liberated one new titrable amino group. Upon treatment with succinic anhydride this one new titrable amino group was lost in accordance with the end group data.

We thus conclude that the positively charged amino group of Ile-16 is not necessary for enzymatic activity in chymotrypsin. The group with pK' of about 9 derived from the kinetics of acylation [5, 6] or on binding of substrate [2–4] awaits further identification.

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