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Conformation of δ-chymotrypsin during catalysis. Chemical reactivity of the isoleucine 16 amino group in the acyl-enzyme intermediate

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There is a considerable body of evidence indicating that a conformational change in the enzyme accompanies the binding of substrates and inhibitors to α- and δ-chymotryosin[1-3]. An acidic group of the enzyme with an apparent pK_a of 9.0 has been shown to participate in this process. This group has been tentatively identified as the isoleucine (Ile) 16 amino group[4]. Kinetic and equilibrium studies have indicated that upon binding of substrates and inhibitors the pK_a of this group changes to a higher value[2,3,5]. Studies in this laboratory have detected shifts of the order of 0.4 to 0.8 pK_a unit when δ-chymotrypsin binds substrates and competitive inhibitors[5]. Substantial changes in the pK_a of this group have been also found upon reaction of the enzyme with diisopropyl fluorophosphate and other inhibitors[4,6].

Except for the related work of Dixon and Hofmann[7] on the rate of deamination of the N-terminal Ile 16 amino group in tosyl- and diisopropylphosphoryl-α-chymotrypsins[7], no studies have been reported on the state of this important group in an acyl-enzyme intermediate in solution. In this communication we report the results of a study on the conformational state of the Ile 16 amino group in indoleacryloyl-succinylated-δ-chymotrypsin, an intermediate in the enzyme-catalyzed hydrolysis of indoleacryloylimidazole, using its reaction with acetic anhydride and HNO_2 [7] as conformational probes.

Succinylated-δ-chymotrypsin was used in these experiments. This derivative has all the ε-amino groups of the lysine residues blocked allowing a very selective reaction of the modifying reagents with the Ile 16 amino group. Furthermore, in contrast to the native enzyme, the deaminated and acetylated products are soluble under the conditions of the reaction. The preparation and kinetic properties of succinylated-δ-chymotrypsin will be described elsewhere[8]. Indoleacryloyl-succinylated-δ-chymotrypsin was prepared by reacting succinylated-δ-chymotrypsin with an excess of indoleacryloylimidazole followed by gel filtration on Sephadex G-25. Deacylation of the acyl-enzyme was followed spectrophotometrically[9] at 340 nm. Enzyme activity was monitored using *N*-acetyl-L-tryptophan methyl ester as substrate[3].

Fig. 1 shows the results of the reaction of succinylated-δ-chymotrypsin and indoleacryloyl-succinylated-δ-chymotrypsin with acetic anhydride at pH 5.8. This reagent rapidly inactivates succinylated-δ-chymotrypsin and the inactivation correlates very closely with the acetylation of the N-terminal Ile 16 amino group. This result is expected from the work of Oppenheimer *et al.*[4]. When the same experiment is carried out with indoleacryloyl-enzyme the results are quite different (Fig. 1, open symbols). After 2 h of treatment, the acyl-enzyme deacylates normally with a $t_{1/2}$ of 280 sec at pH 9.8 and the free enzyme obtained has not lost any of its initial activity. N-terminal analysis carried out by the method of Sanger[10] indicates that in indoleacryloyl-succinylated-δ-chymotrypsin the Ile 16 amino group has not been acetylated.

The results obtained with HNO_2 at pH 3.9 are shown in Fig. 2. In these conditions succinylated-δ-chymotrypsin undergoes a very rapid loss of activity that parallels the disappearance of the N-terminal Ile 16 amino group. When the acyl-enzyme is subjected to the same treatment, it was found that the product deacylates normally ($t_{1/2}$ 280 sec at pH 9.8) and the resulting enzyme is about 95% active. Furthermore, no loss of Ile 16 amino group is detected after 1 h of incubation with HNO_2. Saturating amounts of competitive inhibitors such as benzyl alcohol or *N*-acetyl-D-tyrosinamide in the reaction medium resulted only in a very slight protection, of the order of 15 to 20% and in a substantial loss of Ile 16 amino group. A similar lack of protection by *N*-acetyl-L-tyrosine methyl ester to the inactivation of α-chymotrypsin by HNO_2 has been reported[7].

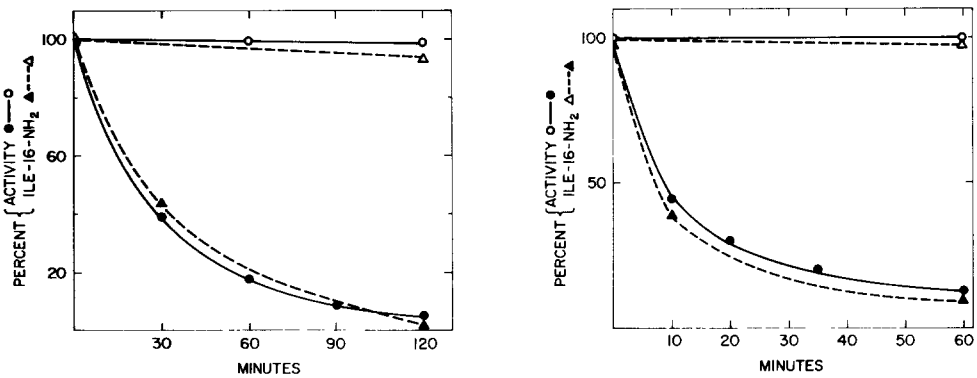

Fig. 1. Reaction of succinylated-δ-chymotrypsin and indoleacryloyl-succinylated-δ-chymotrypsin with acetic anhydride. Proteins (5 mg/ml) were incubated in 0.05 M acetate buffer, pH 5.8, and acetic anhydride (40 μl/ml) was added slowly over a period of 2 h, at 4°. The pH was maintained at 5.8 by automatic addition of 5 M NaOH from a pH-stat. At convenient time intervals aliquots were taken and the enzymatic activity was measured after incubation in 0.1 M glycine buffer at pH 9.8 for 15 min. Aliquots were also taken for quantitative N-terminal analysis. Open symbols: indoleacryloyl-succinylated-δ-chymotrypsin; closed symbols: succinylated-δ-chymotrypsin.

Fig. 2. Reaction of succinylated-δ-chymotrypsin and indoleacryloyl-succinylated-δ-chymotrypsin with HNO_2. Proteins (5 mg/ml) were incubated with 0.4 M HNO_2, pH 3.9 (prepared as described by Gertler and Hofmann[12]), at 4°. At selected time intervals samples were taken for activity and N-terminal analysis, as described in Fig. 1. Open symbols: indoleacryloyl-succinylated-δ-chymotrypsin; closed symbols: succinylated-δ-chymotrypsin.

The results of the experiments reported here show that the acylation of the active site serine in succinylated-δ-chymotrypsin with indoleacryloylimidazole prevents the reaction of the Ile 16 amino group with acetic anhydride at pH 5.8 or HNO_2 at pH 3.9. Whether this effect is the result of a shift in the pK_a of the amino group to a much higher value or due to a steric hindrance to the approach of the reagents in the acyl-enzyme is not yet clear. In any case, we suggest that this group undergoes an appreciable change in conformation upon formation of the acyl-enzyme intermediate. These results are in agreement with those of Dixon and Hofmann[7] who have found that the rate constant for the deamination of the Ile 16 amino group in diisopropylphosphoryl- and tosyl-α-chymotrypsins is 10 and 50 times lower, respectively, than in the free enzyme.

The lack of reactivity of the Ile 16 amino group in the acyl-enzyme is to be related with the postulated salt bridge between this group and the carboxylic group of aspartic acid 194 in crystals of tosyl-α-chymotrypsin[11]. Our results may be taken as evidence for the existence of such an arrangement in the acyl-enzyme in solution.

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