PROPERTIES OF THE ISOLEUCYL AMINO-TERMINUS OF α-CHYMOTRYPSIN

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Summary

The pK_a(app) and reactivity of the isoleucyl amino-terminus of α-chymotrypsin were determined by the method of competitive labelling. In 0.10N KCl at 10.0°C the pK_a(app) is 8.9 and the reactivity towards acetic anhydride is 20% of a normal nucleophile with the same pK_a. Below pH 9.8 the data confirms that the ionization state of this group controls the activity and conformation of α-chymotrypsin. However, above pH 9.8 another structural change occurs which is not dependent on this group.

Introduction

The X-ray crystallographic structure of α-chymotrypsin (Birktoft et al. 1970) indicates that the isoleucyl amino-terminus should have abnormal properties, viz. pK_a and reactivity. The object of the present investigation is to determine these properties in order to resolve the question as to whether the properties of this group can be correlated with the activity of the enzyme. The development of the method of competitive labelling (Kaplan et al. 1969) facilitated this investigation. The unique features of this method are: 1) the values of parameters can be unequivocally assigned to specific residues. 2) the properties determined are for the group on the native protein and not a modified protein.

Materials

3x crystallized, salt-free α-chymotrypsin and 2x crystallized salt-free pepsin were obtained from Worthington Biochemical Corporation.

Acetic-1-C^{14} anhydride, specific activity 5.1 mC/mM and tritiated acetic anhydride 2.09 curies/mM were obtained from Amersham-Searle Corporation. All other reagents and chemicals were high purity preparations obtained from commercial sources.

Methods

Principle of the Method of Competitive Labelling

A small amount of radioactive acetic anhydride is reacted to completion with a large excess of an aqueous solution of protein and internal standard (phenylalanine). Under these conditions the degree of substitution of the protein is negligible in comparison with the total amount of protein so that the reagent reacts predominantly with groups on the native protein. The reaction mixture is fully acetylated with unlabelled acetic anhydride and the acetylated phenylalanine isolated and purified. Suitable enzymatic digestion of the acetylated protein yields peptides containing the labelled groups of interest which can be located in the primary structure by the sequence of the peptide. At any given pH, the specific activities of these peptides relative to acetyl phenylalanine are proportional to their second-order velocity constants. The labelling procedure is repeated at a series of pH's and the pH-dependent and pH-independent second-order velocity constants k' and k are determined from the relationships

$$k'_i = k'_{phe} \times \frac{\text{Specific Activity Peptide i}}{\text{Specific Activity Acetylphenylalanine}}$$

and

$$k' = \frac{k}{1 + \frac{[H^+]}{K_a}}$$

A more detailed account of the method is in preparation (Kaplan et al., in preparation).

Labelling Procedure

25 mg of α-chymotrypsin and 1 μM of L-phenylalanine were dissolved in 250 ml of 0.10N KCl and placed in a cell thermostated at 10.0°C. The pH was adjusted with 2M potassium hydroxide and 0.3 μM of tritiated acetic anhydride (specific activity 2.09 curies/mM) in 25 μl of acetonitrile was added. After five minutes the pH was lowered to 2 with concentrated hydrochloric acid followed by the addition of 2 g of urea. The reaction mixture was left to stand for fifteen minutes. The pH was adjusted to 7 and the reaction mixture completely acetylated with 100 μl of unlabelled acetic anhydride.

Purification of Acetylphenylalanine

The pH of the reaction mixture was lowered to 2 and extracted with ethyl acetate. The ethyl acetate extracts were taken to dryness and the radioactive acetylphenylalanine purified by flat-plate paper electrophoresis at pH 6.5.

Isolation and Purification of the Acetylated Isoleucyl N-Terminal Peptide

The acetylated α-chymotrypsin was thoroughly dialysed, freeze-dried, performic acid oxidized (Hirs, 1956) and digested in 10% formic acid with pepsin. The peptic digest was subjected to high-voltage electrophoresis at pH 6.5 and a radioactive band with a mobility of 0.49 relative to aspartic acid was found to contain the acetylated isoleucyl amino-terminus. A C^{14} labelled marker peptide was used to locate the position of the tritiated

peptide. This band was further purified by paper chromatography with butanol-acetic acid-water-pyridine (15;3;12;10) as solvent, followed by pH 3.5 high-voltage electrophoresis. The peptide was eluted and gave the following amino acid composition after 72 hrs. hydrolysis in 6N hydrochloric acid: $asp_{1.00}$ $gly_{1.16}$ $val_{1.05}$ $ile_{1.04}$. From the known sequence (Hartley and Kauffman, 1966) this peptide corresponds to the acetylated N-terminal peptide Ac-ile-val-gly-asn. The specific activities of the acetylated peptides and acetylphenylalanines from the pH-series were determined by scintillation counting and amino acid analysis.

Table 1

Competitive labelling of the isoleucyl amino-terminus of α-chymotrypsin

pH	Phenylalanine		Isoleucyl amino-terminus	
	cpm/μMx10^{-6}	k'M^{-1}sec^{-1}	cpm/μMx10^{-6}	k'M^{-1}sec^{-1}
7.30	3.24	0.00627	1.55	0.00300
7.88	9.38	0.0234	3.53	0.00881
8.31	14.4	0.0606	4.69	0.0198
8.70	19.3	0.137	5.38	0.0381
8.99	23.9	0.236	4.73	0.0468
9.42	22.1	0.454	3.22	0.0661
9.84	19.0	0.686	2.25	0.0812
10.41	12.3	0.890	1.53	0.111
10.94	6.83	0.965	1.10	0.155
11.64	3.65	0.993	1.20	0.326
12.18	2.95	0.998	0.923	0.312

Results

Table 1 contains a summary of the data for the competitive labelling of the isoleucyl amino-terminus of α-chymotrypsin. The pK_a of the amino group of L-phenylalanine was calculated to be 9.50 at 10°C (Cohn and Edsall, 1943) and the pH-independent second-order velocity constant was taken as 1.

Figure 1 is a plot of k' against pH for the isoleucyl amino-terminus. The solid line is a theoretical curve for a group with pK_a = 8.9 and pH-independent second-order velocity

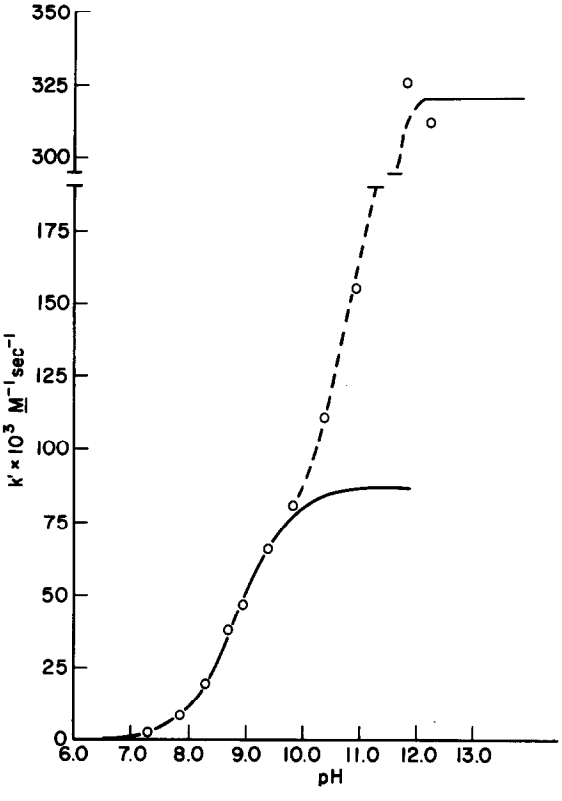

Figure 1. pH-profile of the second-order velocity constant for the reaction of the isoleucyl amino-terminus of α-chymotrypsin with acetic anhydride. The solid line is a theoretical curve for a group with pK_a = 8.9 and a pH-independent second-order velocity constant, k = 0.086 $\underline{M}^{-1}sec^{-1}$ Solvent: 0.10N KCl at 10.0°C.

constant, k, equal to 0.086 $\underline{M}^{-1}sec^{-1}$ relative to phenylalanine. Above pH 9.8 there is a discontinuity in the titration curve with the reactivity of the amino-terminus increasing sharply.

Discussion

Below pH 9.8 the isoleucyl amino-terminus has a $pK_a(app) = 8.9$. The pH-profile of this group (Figure 1) very closely parallels the alkaline pH-activity curve, $pK_a(app) \sim 9$, of α-chymotrypsin (Bender and Kézdy, 1965) and the pH-optical rotation curve, $pK_a(app) = 9.1$, of acetyl-δ-chymotrypsin (Karibian et al., 1968). When compared with a series of sterically unhindered amines (Hartley, 1970), the reactivity of the isoleucyl amino-terminus is only 20% of that expected for an amino group with $pK_a = 8.9$. At pH 12, however, amino-terminus has the reactivity of a normal peptidyl amino group. These observations indicate that below pH 9.8 the deprotonated amino-terminus is not freely accessible to acetic anhydride and that above this pH the molecule denatures and fully exposes the isoleucyl amino-terminus.

In the X-ray crystallographic structure of α-chymotrypsin the protonated isoleucyl amino-terminus is buried in the molecule (Matthews et al., 1967). This suggests that in solution the deprotonated amino-terminus would be unreactive towards acetic anhydride, unless there is a mechanism whereby the deprotonated amino-terminus becomes accessible. Such a mechanism, which incorporates some of the features in a scheme proposed by Oppenheimer et al. (1966), is illustrated in Figure 2. IH^+ and I are conformations of α-chymotrypsin with the protonated and deprotonated amino group "inside". Similarly, OH^+ and O are conformations with the protonated and deprotonated amino group "outside". If it is assumed that O is the only form capable of reacting with acetic anhydride, and that IH^+ is the only enzymatically active form then it follows that

$$K_a(\text{app})(\text{competitive labelling}) = K_a(\text{app})(\text{Kinetic}) = K_o \frac{\left(1 + \frac{1}{K_H}\right)}{\left(1 + \frac{1}{K_L}\right)} .$$

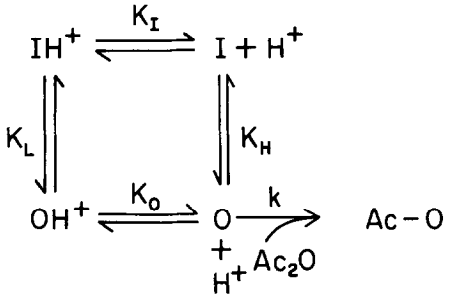

Figure 2. Scheme for the conformational equilibria in α-chymotrypsin at alkaline pH (see text). K_I and K_O are acid dissociation constants and K_L and K_H are equilibrium constants for the formation of OH^+ and O at low and high pH respectively.

The present results from competitive labelling therefore confirm, for the case of α-chymotrypsin, the proposal of Oppenheimer et al. (1966) that the ionization state of the isoleucyl amino-terminus controls the activity and conformation. However, above pH 9.8, α-chymotrypsin undergoes an additional structural change which is not controlled by the isoleucyl amino-terminus.

In the case of porcine elastase the pK_a(app) of the valyl amino-terminus does not parallel the alkaline activity curve (Kaplan et al., 1969 and Kaplan and Dugas, 1969) which indicates that the forms OH^+ and O retain activity and it appears that the same may be true of δ-chymotrypsin (Valenzuela and Bender, 1969). The preliminary results from the competitive labelling of the alanyl-149 amino-terminus of α-chymotrypsin indicate that it is involved in a conformational change on deprotonation of the isoleucyl amino-terminus. Since such a change is not possible

in δ-chymotrypsin, this result supports the proposal of Valenzuela and Bender that, relative to δ-chymotrypsin, the internal splits in α-chymotrypsin may increase the disruptive effect of deprotonating the isoleucyl amino-terminus. Further experiments are in progress to test this hypothesis.

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