THE REACTIVITY OF CARBOXYL GROUPS IN CHYMOTRYPSINOGEN

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1. Introduction

Recent work from this laboratory reported the functional and structural consequences of the modification of carboxylates in chymotrypsinogen (ChTg), chymotrypsin (ChTi), trypsinogen (Tg) and trypsin (Ti) [1] with glycine ethyl ester (GEE) under the influence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) by the method of Hoare and Koshland [2]. The results of a similar and independent study were published at about the same time by Carraway et al. [3]. We present in this paper complementary information and the results of our sequence studies*.

2. The modification of surface carboxylates in chymotrypsinogen at different pH. influence on the activation of the zymogen

Fig. 1a shows the influence of pH on the total number of glycine residues incorporated into the chymotrypsinogen A molecule after 16 hr at 25°C. Between pH 5.4 and 6.6 10 carboxylates (ChTg"10") are modified easily. Between pH 3.5 and 4, 13 carboxylates are modified (ChTg"13"). Previous kinetic results at pH 4 [1] had also indicated the presence of at least 2 families of surface carboxylates with different reactivities. The modification of 3 more carboxyls does not change the potential activity of the zymogen (fig. 1a).

Hoare and Koshland's method is usually employed at pH 4.75 [2]. Fig. 1A shows that the chemical modification can be carried out conveniently at much higher pH values.

The modified zymogen (ChTg"13") is activated more rapidly by trypsin [3,4]. A more detailed kinetic analysis is presented in fig. 1B and 1C. It indicates that the modification of surface carboxylates decreases K_m by a factor of 20 and increases the maximal rate (k_{cat}) by a factor of 10. At low concentrations, ChTg"13" is activated 200 times faster than the native precursor.

3. The side-chain of Asp-194 is already masked in the zymogen molecule

The most recent crystallographic studies of αChTi have shown that the β-carboxyl function of Asp-194 forms with the α-amino group of Ile-16 an ion pair which stabilizes the native conformation of the enzyme [5]. Physicochemical results suggested that this residue is already masked in the zymogen molecule [4,6]. The chemical proof was obtained as follows.

1 g of ChTg A in 200 ml was treated with 27.8 g of GEE and 6.40 g of EDC at pH 4, 25° (pH-stat). EDC was added in three parts at time 0, 3 hr and 16 hr. The reaction is carried out during 24 hr. After this time, half of ChTg A has precipitated and is discarded. The soluble part is dialyzed and lyophylised to give a chymotrypsinogen molecule which has incorporated 13 glycine residues (ChTg"13").

ChTg"13" was then denaturated in 8 M urea pH 2 during 15 min and treated again with carbodiimide and ^{14}C-GEE, in order to bind covalently radioactive glycine residues on buried carboxylates. A maximum

* Many of the results described here have been presented at the "Symposium on Structure-Function Relationships of Protectic Enzymes" (Copenhagen, June 1969).

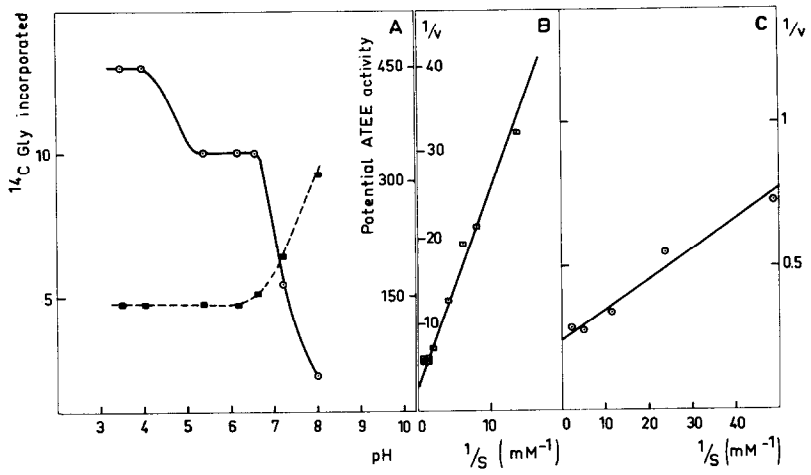


Fig. 1A. Number of Gly ^{14}C ethyl ester incorporated into ChTg A at different pH values. Conditions were as follows: ChTg A 2 mg/ml, GEE 1 M, EDC 6.25 X 10^{-2} M during 16 hr at 25° in a pH-stat (Radiometer). ChTi activity (μM/mn/mg) of the activated zymogen was measured by the pH-stat method with acetyl-L-tyrosine ethyl ester (ATEE) 10^{-2} M at pH 8, 25°, NaCl 0.2 M. The maximal activity value obtained from the unmodified zymogen is 500 μM/mn/mg. 1B. Lineweaver-Burk plot for the activation by trypsin of native ChTg A. pH 8, 1°C, ionic strength 0.3 [12]. v: μM ChTi/mn/mg Ti, K_m = 1.09 mM, k_{cat} = 0.18 sec^{-1}. 1C. Lineweaver-Burk plot for the activation of ChTg"13". pH 8, 1°C, ionic strength 0.1. v: μM ChTi"13"/mn/mgTi. K_m = 0.055 mM, k_{cat} = 1.8 sec^{-1}.

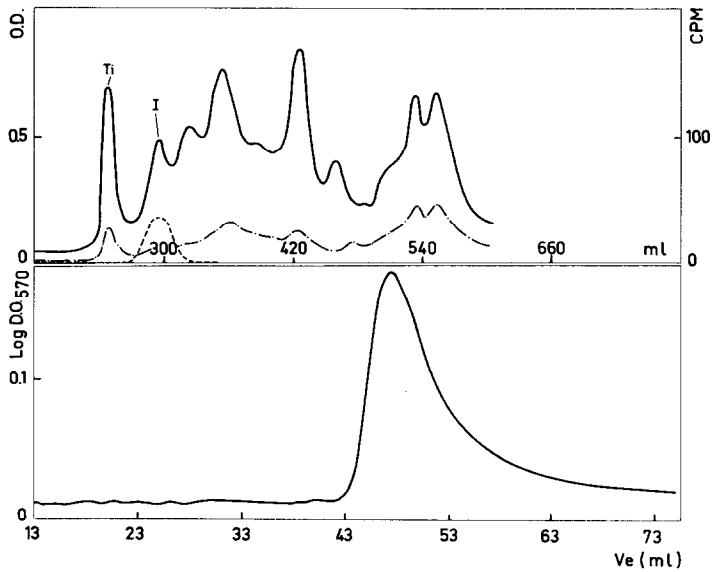
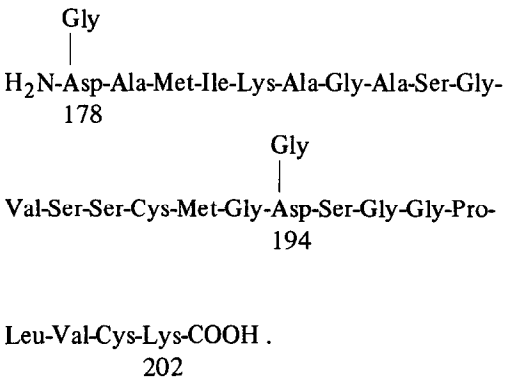


Fig. 2a. G 25 (fine) chromatography of tryptic peptides. Column 2 X 200 cm. Elution by HCl N/100 containing NaCl 0.1 M. The insoluble core [13] was previously eliminated by a centrifugation at 13,000 rpm during 15 min. — optical density 230 mμ; –.– optical density 280 mμ; - - - - - radioactivity. Peak 1 corresponds to the peptide with Asp-194 (Asp_{178}- - -Lys_{202}). 2b. Purification of peptide I on Dowex 50 X 8 (0.9 X 110 cm) at 40°C. The elution is carried out with a pH-gradient from 2.9 to 6 (pyridine-formic acid buffer). The outflow of the peptide was measured after reaction with ninhydrin by the optical density at 570 mμ.

of 3 carboxylates is not readily available to EDC in the native protein as checked both by radioactivity measurements and amino-acid analysis. After reduction with mercaptoethanol and carboxymethylation by iodoacetic acid the protein is digested by trypsin. The tryptic peptides are chromatographed on Sephadex G 25 (fig. 2a). The first eluted peptide is purified on Dowex 50 X 8 (fig. 2b). The sequence of this peptide as judged from the amino-acid composition and the full sequence determined by Hartley and Kauffman [7] is:



2 glycine residues are covalently bound to Asp-194 and 178. Only 1.2 radioactive glycine has been introduced.

The peptide was then treated with dinitrofluorobenzene and attacked with cyanogen bromide in HCl 0.1 N during one night. Cyanogen bromide will split beside methionines 180 and 192 [8]. After butanol extraction of the DNP-Asp-Ala-Homoser peptide it was found that the radioactive glycine residue is covalently bound to Asp-194. The side-chain of Asp-194, which will form the salt bridge in the active enzyme, is already buried and inaccessible in the zymogen molecule. An identical conclusion has been obtained independently by Carraway et al. [3]. This side-chain is also probably the one whose unmasking at acidic pH controls, in the zymogen and in the enzyme [4,5], a conformational change which destroys the proper geometry of the active center [4]. This conformational change involves a distortion or destruction of part of the antiparallel pleated-sheet structure [9] as should be expected since some of the elements of the active center are part of a β-structure of the antiparallel type [10].

4. The accessibility of the α-carboxylic function of the C-terminal asparagine residue

The α-carboxylic function of the C-terminal asparagine residue should not be expected to be particularly reactive with carbodiimides. The pK of an α-carboxylic function is much lower than that of β or γ-carboxylic functions. Furthermore it has been reported [11] from an inspection of the model of α-chymotrypsin obtained by X-ray diffraction [5], that the α-carboxyl of Asn-245 forms an ionic bond with Lys-107 which might be important as a protection against carboxypeptidase digestion. The accessibility of α-carboxyl-245 was checked as follows. A comparison was made between native ChTg and ChTg"13". They were both reduced, carboxymethylated and attacked by carboxypeptidase A at pH 8 at 30°C during one night. Products were analysed on an Unichrom Beckman analyser and it was found that practically no attack occurs on ChTg"13" as compared to the native protein. For example amino-acid analysis indicates that 0.92 mole of Leu-244 and 1.07 mole of Thr-243 are liberated per mole of native ChTg. The same experiment gives 0.073 mole of Leu-244 and 0.079 mole of Thr-243 per mole of ChTg"13". This experiment shows clearly that the C-terminal α-carboxylic function was mofidied with a yield of about 93%. Since ChTg"13" can be easily transformed into an active chymotrypsin which has preserved 50% of its activity [1], with no loss in the percentage of its active center, it is clear that the α-carboxylic group of Asn-245 is essential neither for the structure nor for the function of the enzyme. It has been shown previously that the physicochemical properties of ChTg"13" were slightly different from those of native ChTg. The molecule has different ORD properties and is less resistant to thermal denaturation [1]. One reason for this increased fragility might be the modification of the α-carboxylic acid: the electrostatic bond Asn-245-Lys-107 is no longer possible and a conformational change might have been introduced in a part of the ChTg molecule which is important for its stabilisation since its contains the largest helical segment [10]. It has been found previously that the covalent modification of α-carboxyls of Leu-13 and Tyr-146 in α-ChTi does not change its catalytic properties [1].

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