ON THE STRUCTURAL AND FUNCTIONAL ROLE OF CARBOXYLATES IN CHYMOTRYPSINOGEN A A COMPARISON WITH CHYMOTRYPSIN, TRYPSINOGEN AND TRYPSIN.

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The most recent crystallographic studies of chymotrypsin (ChTi) suggest the presence of at least 2 important carboxylates in this molecule. The side-chain of Asp 194 forms with the α-amino group of Ile 16 an ion pair which stabilizes the native conformation of the enzyme (1). Asp 102 forms with His 57 and Ser 195 a hydrogen bonded system which should apparently increase the nucleophilicity of the serine oxygen (2). Aboderin and Fruton (3) previously noticed an inactivation of ChTi with diphenyldiazomethane. Acidic conformational changes controlled by the unmasking of abnormal carboxylates have been found with chymotrypsinogens (ChTg) A and B, ChTi, trypsinogen (Tg) and trypsin (Ti) (4,5).

ChTg A and B and Tg have an apparently identical specific site for Ca^{2+} which also exists in ChTi and Ti (5,6). The binding of Ca^{2+} on this site induces a stabilization of the conformation of these proteins which prevents the formation of inert proteins in the course of Tg activation and protects Ti against autolysis. This Ca^{2+} site is beleived to be composed of carboxylate functions (6). In the particular case of Tg one must add to the important carboxylates the unusual N-terminal sequence $(Asp)_4$ (7). For Ti it can be expected that the specificity site which must recognize the specific positively charged substrates also includes at least one carboxylic function. We study in this paper the influence of the chemical modification of carboxylates in ChTg, ChTi, Tg and Ti with glycine ethyl ester under the influence of 1-ethyl-3 dimethylaminopropyl carbodiimide (EDC) by the method of Hoare and Koshland (8). There are 14 carboxylates in ChTg A (9). Fig. 1 A presents the kinetics of the reaction with ChTg A. In these conditions 13 carboxylates are modified (ChTg 13). The same reaction in 8 M urea

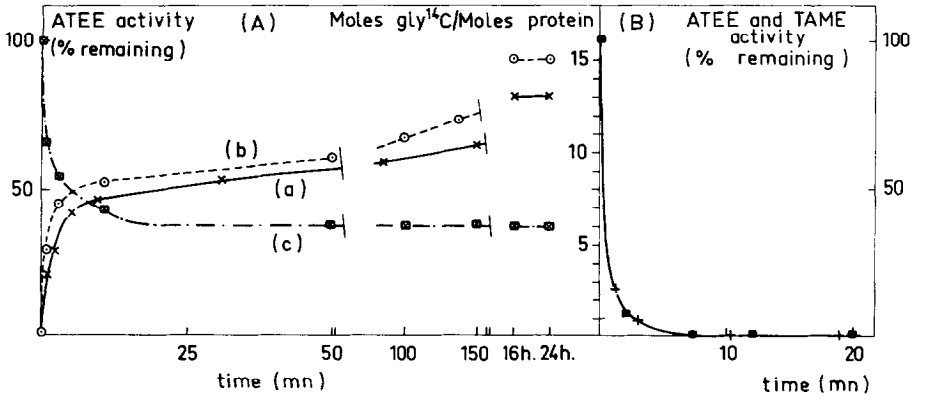

Fig. 1 A. Kinetics of the modification of carboxylates in ChTg (a) and ChTi (b). Protein concentration 5 mg/ml, glycine ethyl ester (GEE) 1 M, EDC 6.25 x 10^{-2} M. The reaction is carried out in a pH-stat (Radiometer) at pH 4,25°. The number of Gly ^{14}C incorporated was counted in a Packard Spectrometer. The maximal number and the specificity of the reaction were checked by amino acid analysis. (c) loss of activity of α-ChTi in the course of the modification. ChTi activity was measured by the pH stat method with acetyl-L-tyrosine ethyl ester (ATEE) at pH 8,25°, NaCl 0.2 M. It was demonstrated by hydroxylamine treatment (14) that no glycine residue was incorporated on tyrosine side-chains. Fig. 1 B. Modification of carboxylates in α-ChTi (■) and Ti (+) in 8 M urea. Other conditions are the same as in fig. 1 A. Ti activity was measured with tosyl-L-arginine methyl ester (TAME). The titration of the active center of Ti with the pancreatic trypsin inhibitor follows closely the loss of activity.

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involves the modification of all the 14 carboxylates of the molecule (ChTg 14). ChTg 13 and ChTg 14 are homogeneous in chromatography and disc-electrophoresis. This experiment suggest that 13 carboxylates are on or near the surface of the protein and readily accessible to EDC. The last function is in the hydrophobic interior of ChTg A and previous denaturation of the native structure is necessary to make it accessible to the reagent. A comparison of ORD spectra is given in fig. 2 A, B, C. Native ChTg presents 2 minima at 232 and 222 mμ which characterize the active structure. After urea denaturation a single band appears at 218 mμ which is then typical of the unfolded structure. There is a complete disappearance of the band at 232 mμ. 3 minima are observed with ChTg 13 at 232, 222 and 213 mμ. This molecule thus appears to be slightly more unfolded than native ChTg. The transition curve of ChTg 13 (fig. 2D) shows that this molecule, although more fragile to the thermal denaturation than ChTg, still presents an ordered structure which can be destroyed at high tem-

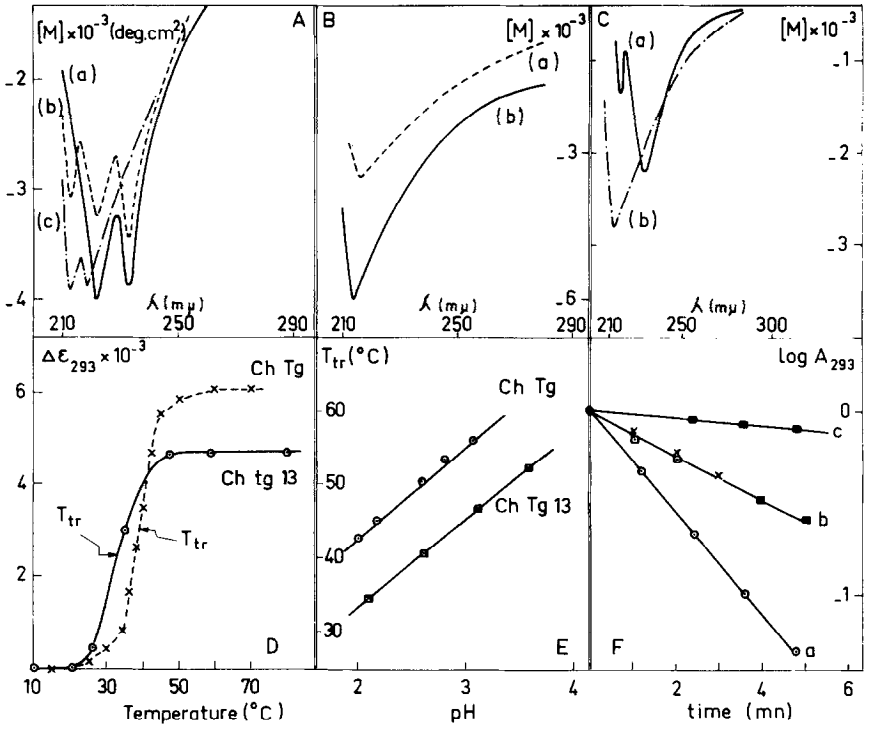

Fig. 2 A. ORD spectra (Fica spectropolarimeter) of native ChTg (a), ChTg 13 (b), ChTg 14 (c). pH 2, 15°. Fig. 2 B. ORD spectra of ChTg (a) and Tg (b) in 2.7 M urea, pH 2, 15°. Fig. 2 C. ORD spectra of native Tg (a) and Tg 11 (b), pH 2, 15°. Fig. 2 D. Thermal denaturation of ChTg (pH 2) and ChTg 13 (pH 2.1) followed by the unmasking of Tyr and Try at 293 mμ (4). Fig. 2 E. Variation of T_{tr} with pH for ChTg and ChTg 13. Fig. 2 F. First-order denaturation of ChTg and ChTg 13 in urea followed spectrophotometrically at 293 mμ (4). ChTg in 8.7 M urea, pH 8.5, 25°, NaCl 0.1 M, without Ca^{2+} (a) and in Ca^{2+} 10^{-2} M (c). ChTg 13 in 8 M urea, pH 8.5, 25°, NaCl 0.1 M, without Ca^{2+} (x) and in $Ca^{2+}10^{-2}$ M (□) (b). $A_{293} = \frac{\Delta\varepsilon_{293\ max} - \Delta\varepsilon_{293}}{\Delta\varepsilon_{293\ max}}$. $\Delta\varepsilon_{293\ max}$ and $\Delta\varepsilon_{293}$ correspond to the total denaturation and to the denaturation at time t.

peratures. The variation of T_{tr} with pH for ChTg A and B was interpreted previously as resulting from the unmasking of the abnormal carboxylate buried in the interior of the native structure (5). Since T_{tr} decreases with pH for ChTg 13 as well as for ChTg (fig. 2E), we have the physico-chemical indication that the structural carboxylate is still buried in ChTg 13 at neutral pH. The nitration with C $(NO_2)_4$ (10) shows that in ChTg 13, as in ChTg (11) 2 of the 4 tyrosines are accessible to the reagent. Chemical modi-

fication with 5-nitrobenzyl 2-hydroxy bromide (12) shows that the 8 tryptophans of ChTg 13 are masked to the reagent as they are in ChTg A and B (5). Finally, since ChTg 13 can be activated at the same rate as ChTg, the sequence Glu_{20}-Glu_{21} located near the strategic Arg_{15}-Ile_{16} (9) bond has no crucial role in the activation mechanism.

ChTg 14 presents all the properties of an unfolded molecule. Its ORD spectrum (fig. 2A), like that of urea-denatured ChTg, presents no trough at 232 mμ typical of the native structure. Urea induces no change of the ultra-violet spectrum at 293 mμ at it does for ChTg and ChTg 13 (fig. 2F) ; this indicates that all tyrosines and tryptophans are unmasked in ChTg 14.

The Ca^{2+} -site no longer exists in ChTg 13. This molecule has lost the property of native ChTg to be stabilized by Ca^{2+} against urea denaturation (fig.2F). It is clear then that some or all of the ligands which bind Ca^{2+} to native ChTg are carboxylates. Good candidates would be Asp 72, Glu 78 and Asp 154 which occur in homologous positions in Tg and ChTg A and B (9). The enzymatic activity of ChTi 13 obtained from the activation of ChTg 13 is decreased by a factor of about 2 for all substrates when compared to ChTi (table I). As expected, the activation of ChTg 14 is impossible ; addition of Ti to the zymogen solution gives rise to no chymotryptic activity. The production of ChTi 15 by modification of carboxylates in native α-ChTi is shown in fig. 1A. First, in the course of a rapid modification of about 8 carboxylates ($t_{1/2}$ = 40 sec.) the enzymatic activity decreases by about 60%. A slower modification of 7 other carboxylates brings no further loss of activity. ChTi 15, and ChTi 13 obtained from the activation of the modified zymogen, have similar kinetic properties (table I). Consequently, the modification of the α-carboxylic functions of C-terminal Leu 13 and Tyr 146 induce no loss of activity. In ChTg also (fig. 1A) there are 2 types of carboxylates available to EDC. The first group of about 7 functions is modified rapidly, the 6 other functions are modified more slowly. 16 glycine residues are covalently bound to carboxylates in α-ChTi (ChTi 16) when the reaction is carried out in 8 M urea. ChTi 16 has no activity (fig. 1 B). All these facts taken together show apparently that there exist in ChTg as in ChTi a carboxylate completely masked to EDC which plays a considerable structural role since its chemical modification involves very drastic changes of the conformation of both proteins. Sequence work is being carried out to localize this important residue.

TABLE I

Enzyme	Substrate	v (μM/mn/mg)	V_m (μM/mn/mg)	K_m (mM)	% of active center[d]
α ChTi	ATEE[a]		525	3.5	100
	A Phe NA[b]	0.014			
	Hb[c]	1.83			
α ChTi 13	ATEE		277	2.5	95
	A Phe NA	0.006			
	Hb	0.99			
α ChTi 15	ATEE		280	4.5	92

(a) pH 8, 25°, NaCl 0.2 M ; (b) Acetyl-DL-Phe-p nitroanilide (A Phe NA) 10^{-4}M in a solution containing 3% of dimethyl formamide, pH 8, 25°, NaCl 0.1 M. The reaction was followed spectrophotometrically at 388 mμ. (c) Haemoglobin (Hb) 10 mg/ml in 3 M urea, pH 9, 25°. The reaction was followed in a pH-stat (Radiometer). (d) Measured with p.nitrophenyl acetate 10^{-3}M by the burst of p.nitrophenol, pH 7.5, 25°, NaCl 0.1 M.

If it is Asp 194, as seems reasonable to us (5), then we should conclude that the chemical modification of Asp 102 brings only a slight change of the chymotryptic activity.

As for ChTi, blocking of all the carboxylates of unfolded Ti in 8 M urea results in a total loss of activity (fig. 1 B). As expected, in the same conditions, all 11 carboxylates are modified in Tg. Tg 11, like ChTg 14, presents all the properties of a denatured species. Its ORD spectrum is very similar to that of Tg in urea (fig. 2 B, 2 C) and experiments of thermal and urea denaturations show that all tryptophan and tyrosine residues are unmasked. These results confirm the physico-chemical conclusions (4) that there exist in Tg and Ti, as in ChTg and ChTi carboxylic functions with a key structural role. Partial modification of carboxylates in Tg gives a precursor form which has lost the property of autoactivation but still gives rise to active Ti (13). We have observed that this enzyme can no longer be stabilized against autolysis by Ca^{2+}. This experiment, like those presented in fig 2 F, shows that for this family of proteins the Ca^{2+} ligands are carboxylates.

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